REPORT DOCUMENTATION PAGE

AFRL-SR-BL-TR-98-

0607

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE
		FINAL 15 Ap

4. TITLE AND SUBTITLE
Mechanism(s) of Molecular Responses in Mammalian Cells Exposed to Physical Agents (Alone and in Combination

5. FUNDING NUMBERS
F49620-95-1-0337
61102F
2312/AS

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Dr Walter Kozumbo

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release;
distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Among the many reported protective effects of melatonin, including immunomodulation during acute stress, endogenous hydroxyl radical scavenging, and inhibition oftumor growth in experimental animal models, our laboratory has observed radioprotective effects of melatonin in normal human lymphocytes. As a preliminary study, before examining the radioprotective effect of melatonin in proliferating 293S human embryoni kidney cells, we have investigated the effect of melatonin treatment on several normal cellular processes. These include cell growth, viability, metabolic activity and cell cycle phase distribution. When cell viability was measured at the selected time points by the trypan blue dye exclusion method, no significant cell death was observed Even after the 2 mM melatonin treatment for 72h, the viability remain at 98%. When the metabolic activity was determined at 24, 48 and 72h using the colorimetric MTT assay, no significant change in metabolic activity was observed. Even with the 10 mM melatonin treatment for 72h, the metabolic activity was similar to that of control. When cell cycle analysis was performed by flow cytometry, no marked difference in cell cycle distribution was observed. Melatonin at 2 mM concentration did slightly increas the percentage of SPF phase cells compared to controls after 72h. This study indicate

14. SUBJECT TERMS			15. NUMBER OF PAGES
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT (U)	18. SECURITY CLASSIFICATION OF THIS PAGE (U)	19. SECURITY CLASSIFICATION OF ABSTRACT (U)	20. LIMITATION OF ABSTRACT (UL)

that when 293S cells are treated with concentrations of melatonin up to 2 mM, a concentration which was previously found to protect against radiation-induced chromosome aberrations in human lymphocytes (Vijayalaxmi et al, 1995), no significant alterations in the assayed cellular functions in the 293S human embryonic kidney cells occurred.

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Annual and Final Report
U.S. Air Force Office of Scientific Research
Grant No. F49620-95-1-0037

"Mechanism(s) of Molecular Responses in Mammalian Cells Exposed to Physical Agents (Alone and in Combination)

Project Period: 1 September, 1997 - 14 April, 1998

Date of Submittal: 30 April, 1998

2. OBJECTIVES

The objectives of this research did not substantially change from those described in an our Annual Report submitted in August, 1997, with the addition of additional activity undertaken in the development of techniques for detection of nitrated proteins, as described in our Annual Report of August, 1997.

3. STATUS OF EFFORT

The research activity of this project was very productive, with continued interaction with our colleagues in the Directed Energy Division of the U.S. Air Force Research Laboratory at Brooks AFB, Texas. The research data collected resulted, since our last annual report, in further presentations at national and international scientific meetings, and additional publications in peer reviewed journals. Our collaborative efforts with colleagues in the U.S. Army Medical Rsearch Detachment at Brooks AFB, and at the University of Texas Health Science Center at San Antonio, allowed for continued investigation of the role of melatonin in protecting genetic material against radiation damage, and molecular level responses at lower radiation levels.

4. ACCOMPLISHMENTS/NEW FINDINGS

The Accomplishments/New Findings of the grant investigators for this final project period are presented by investigator, both to indicate the level of their individual activity, and to demonstrate the continued application of their expertise. The research findings (since the last report) of Dr. Natarajan and Dr. Vijayalaxmi are presented. Dr. John Bruno did not directly participate in this phase of the grant, since he had transferred his office and research activity to Brooks AFB under an IPA agreement between the U.S. Air Force and the University of Texas Health Science Center at San Antonio. Dr. Bruno is an Assistant Professor at UTHSCSA, and continued to attend, on a regular basis, the weekly Radiation Biology Group meetings at UTHSCSA.

Ultrawideband electromagnetic radiation (UWBMWR) and genetic damage in the peripheral blood and bone marrow cells of mice.

[Vijayalaxmi, Lead Investigator]

This is a collaborative research project initiated with Dr. Ronald L. Seaman, McKesson BioServices, and the Microwave Bioeffects Branch, US Army Medical Research Detachment, Brooks Air Force Base, Texas.

Objective: To investigate the extent of genetic damage in the peripheral blood and bone marrow cells of mice exposed to UWBR for 15 minutes.

Experimental protocol: CF-1 male mice were exposed to UWBMWR for 15 minutes at an estimated whole-body average specific absorption rate of 37 mW/kg. Groups of control and positive control mice injected with mitomycin C were also included in the study. After various treatments, half of the

mice were sacrificed at 18 hours, and the other half at 24 hours. Peripheral blood and bone marrow smears were made on microscopic slides and stained with acridine orange. Coded slides were examined to determine the extent of genotoxicity, as assessed by the presence of micronuclei (MN) in polychromatic erythrocytes (PCEs). The results were decoded and analyzed for statistical significance.

Results: The percentages of PCEs and the incidence of MN/2000 PCEs in both tissues in mice sacrificed at 18 hours were similar to the frequencies observed in mice terminated at 24 hours. There were no significant differences in the percent PCEs between control and the mice±UWBMWR exposure; the group mean values (± standard deviation) were in the range of 3.1±0.14 to 3.2±0.23 in peripheral blood, and 49.0±3.56 to 52.3±4.02 in bone marrow. The mean incidence of MN/2000 PCEs in control and mice±UWBMWR exposure ranged from 7.7±2.00 to 9.7±2.54 in peripheral blood, and 7.4±2.32 to 10.0±3.27 in bone marrow. Pairwise comparison of the data did not reveal statistically significant differences between controls and mice±UWBMWR exposed groups (excluding positive controls).

Conclusion: Under the experimental conditions tested, there was no evidence for excess genotoxicity in both peripheral blood and bone marrow cells of mice exposed to ultrawideband microwave radiation.

Publication: The data have been written-up and the manuscript is provisionally accepted for publication in the International Journal of Radiation Biology, 1998. A copy of the revised paper is enclosed in the Appendix.

Some of the results have also been presented as an abstract in the proceedings of the Twentieth Annual Bioelectromagnetics Society Meeting held at St. Pete Beach, Florida, June 7-11, 1998. A printed abstract is enclosed in the Appendix.

Protection from radiation-induced primary DNA damage in peripheral blood lymphocytes obtained from human volunteers following in vivo administration of melatonin.

[Vijayalaxmi, Lead Investigator]

Objective: To examine the ability of a single oral administration of melatonin to human volunteers to protect radiation-induced primary DNA damage in their peripheral blood lymphocytes.

Experimental protocol: Peripheral blood samples were drawn from four separate human volunteers at 5-10 minutes before (0 hour) and at 1 and 2 hours following a single oral ingestion of a 300 mg dose of melatonin. After each collection: (a) the concentration of melatonin was determined in the serum, and (b) aliquots of whole blood samples were exposed in vitro (on ice, to prevent the rejoining of DNA strand breaks) to 100 cGy gamma radiation. Immediately, lymphocytes were processed for the alkaline comet assay. The extent of primary DNA damage, i.e., single strand breaks and alkali labile lesions, was assessed from the length of DNA migration and fluorescence intensity of migrated DNA in the comet tail.

Results: For each volunteer, the results showed a significant increase in concentration of melatonin in the serum and in the leukocytes in the blood samples collected after the oral dose of melatonin, as compared with the blood samples collected before melatonin ingestion (0 hour). The lymphocytes in the blood samples collected at 1 and 2 hours after melatonin ingestion and exposed in vitro to 100 cGy gamma radiation exhibited a significant decrease in the extent of primary DNA damage, as compared with similarly irradiated lymphocytes from the blood samples collected before melatonin ingestion. The extent of melatonin-associated decrease in primary DNA damage did not correspond with the decrease reported earlier in the incidence of chromosomal aberrations and micronuclei. The latter assays required an additional post-irradiation incubation of the cells at 37±1°C for 48 and 72 hours, respectively.

Publication: The results have been written-up and the manuscript entitled "Melatonin reduces gamma radiation-induced primary DNA damage in human blood lymphocytes" by Vijayalaxmi, RJ Reiter, TS Herman and ML Meltz has been published in Mutation Research, 397, 203-208, 1998.
A copy of the published paper is enclosed in the Appendix.

Melatonin and protection from acute whole-body irradiation in mice.
[Vijayalaxmi, Lead Investigator]

This study has been supported by a grant from the Armed Forces Radiobiology Research Institute, Bethesda, Maryland.

Objective: To examine whether the mice which were treated with melatonin prior to acute whole-body exposure to gamma irradiation at the expected $LD_{50/30}$ dose exhibit a higher survival rate as compared with similarly irradiated mice which were not pre-treated with melatonin.

Experimental protocol: Two separate experiments using a total of 160 mice were conducted. Each experiment had 10 mice in each of eight groups. A randomized block design based on body weights was used to distribute the mice into the following separate groups: (1) control, (2) 815 cGy whole-body gamma radiation, (3) solvent control, soybean oil, (4) solvent + 815 cGy whole-body gamma radiation, (5) melatonin, 125 mg/kg body weight, (6) melatonin, 125 mg/kg body weight + 815 cGy whole-body gamma radiation, (7) melatonin, 250 mg/kg body weight, (8) melatonin, 250 mg/kg body weight + 815 cGy whole-body gamma radiation

Mice in groups 1, 3, 5 and 7 served as the controls for the experimental mice in groups 2, 4, 6 and 8, respectively. One hour before the start of each experiment, all mice were transferred to a laboratory near the ^{137}Cs GammaCell-40 Irradiator (Atomic Energy of Canada Ltd.) facility. Control mice in group 1 were untreated and unirradiated. Mice in group 2 were exposed to an acute whole-body gamma radiation dose of 815 cGy (the expected $LD_{50/30}$ dose). The dose rate used was 103 cGy/minute. Mice in groups 3-8 were given an intraperitoneal injection of 100 μl of either the solvent (soybean oil) or melatonin (freshly prepared as a uniform suspension in soybean oil). One hour after the injections, mice in groups 4, 6 and 8 were exposed to an acute whole-body gamma radiation dose

of 815 cGy. One hour after irradiation, all mice were returned to the animal facility. They were inspected twice daily, at least 6 hours apart (before 1000 hr and after 1400 hr) for morbidity and mortality. All mice which were alive on day 31 after radiation exposure were euthanized using halothane.

Results: The results indicated 100% survival for unirradiated and untreated control mice, and for mice treated with melatonin or solvent alone. Forty-five percent of mice exposed to 815 cGy radiation alone, and 50% of mice pre-treated with solvent and irradiated with 815 cGy were alive at the end of 30 days. In contrast, 60% and 85% of irradiated mice which were pre-treated with 125 mg/kg ($p=0.3421$) and 250 mg/kg melatonin ($p=0.0080$), respectively, were alive at 30 days. These results indicate that melatonin at a dose as high as 250 mg/kg is non-toxic, and that high doses of melatonin are effective in protecting mice from the lethal effects of acute whole-body irradiation.

The data have been written-up for publication. The manuscript entitled "Melatonin and protection from whole-body irradiation: Survival studies in mice" by Vijayalaxmi, Martin L. Meltz, Russel J. Reiter, Terence S. Herman and Sree Kumar K, is currently under review. A copy is enclosed in the appendix.

Radioprotective antioxidant compounds interfere with *In Vitro* Cytoxicity Assay

[M. Natarajan, Lead Investigator]

Objective: To determine whether radioprotective anti-oxidants influence *the in vitro* cytotoxicity assay using 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetraziliumbromide (MTT) reagent.

Methodology:

Antioxidant Solution Preparation

(a) Ascorbic Acid, Dithiothritol (DTT), N-aetyl L-cysteine (NAC), Oxothioziludine decaboxylase (OTC), 2-Mercaptoethanol (2-ME), Pyrolidine di-thiocarbomate (PDTC):

The 0.5 or 1M stock solutions of ascorbic acid, DTT, NAC and OTC, 2-ME and PDTC were prepared in 1x phosphate buffered saline (PBS) and stored in aliquots at -20°C. From each stock solution, Working Solutions at final concentrations of 0.001, 0.01, 0.1 and 1 mM were prepared freshly before addition into the culture medium.

(b) α-Tocopherol succinate and Melatonin:

Stock solutions of 0.5M α-tocopherol succinate and melatonin were prepared in absolute ethanol. From each stock solution, working solutions at final concentrations of 0.001, 0.01, 0.1 and 1 mM were freshly prepared before addition into the culture medium. The highest concentration of 1mM of either melatonin or α-tocopherol (used in this study) resulted in a final concentration of not more than 0.2% (Vol/Vol) of absolute ethanol.

(c) Iodoacetic acid:
A stock solution of 1M iodoacetic acid was prepared in PBS. Working solutions of 5, 10, 20 and 40 mM were freshly made before addition into the culture medium.

MTT Assay
The MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-di-phenyltetrazolium bromide] assay was performed by two methods: (a) as described originally by Mosmann (1983); and (b) using a currently available commercial kit following the manufacturers protocol (Promega, Madison, WI). Different concentrations of the antioxidant compounds were added to complete RPMI growth medium. To evaluate the cytotoxicity of the anti-oxidant compounds, the MTT assay (Hussain, et al 1993) was performed by incubating 200µl of lymphocytes (1 x 10^5 cells/ml) with various concentrations of the reagents in a 96-well plate (Promega, Madison, WI). After 6 h of incubation, 20 µl of sterile MTT (5 mg/ml) dye (Sigma, St. Louis, MO) was added. The cells were then incubated at 37°C in a humidified incubator for another 4 h. After removing a 120 µl of the medium from the top, 0.1 ml of acidic isoproponal (0.04 M HCL in isoproponal) were added and thoroughly mixed. Spectrophotometric absorbance at 540 nm (for formazan dye) was measured using a plate reader (Dynatech Laboratories, Chantilly, VA).

Status:

The MTT assay has been widely used to quantitate cellular toxicity and proliferative response after treatment with various physical and chemical agents. In living cells, mitochondrial succinate dehydrogenase reduces the substrate MTT to produce blue formazon crystals; these are then dissolved and absorbance is measured at 570 nm. Many relevant modifications have been carried out in numerous laboratories to improve the sensitivity and reliability of the assay. We have found that simple thiol compounds, such as N-acetyl cysteine, glutathione monoethyl ester, ascorbic acid, α-tocopherol, and 2-mercaptoethanol, which have been used as radioprotectors and also as immunomodulators, reduce MTT and result in similar blue color readings as those resulting from metabolic activity. In contrast, L-2-oxothizolidine-4-carboxylate (OTC), which is cleaved enzymatically only inside the cell and known to provide reducing equivalents after intracellular conversion, did not affect the results of the MTT assay. It thus appears that free reactive thiol compounds added to the culture medium can rapidly reduce MTT. Therefore, it is very important to be aware of the free thiol content of a compound before testing it for either proliferative or cytotoxicity activity using the MTT assay. A manuscript describing this research is in preparation.

EFFECT OF MELATONIN ON CELL GROWTH, METABOLIC ACTIVITY AND CELL CYCLE DISTRIBUTION

[M. Natarajan, Lead Investigator]

Objective:
To determine the effect of melatonin treatment on normal cellular processes.

Methodology:

Cell Culture: Adenovirus type V transformed human embryonic kidney (293) cells (obtained from ATCC, Rockville, MD) were cultured in complete Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine and 10 mM HEPES (Mediatech, Inc., Herndon, VA) and supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 50μg/ml gentamicin. The cells were sub-cultured twice weekly by diluting the cell suspension to 2.5 x 10^5 cells/ml. For all experiments, the cells were grown to a maximum cell density of approximately 1 x 10^6 cells/ml on the day of the experiment.

Melatonin Treatment: A 0.5 M stock solution of melatonin (Sigma, St Louis, MO) was prepared in 200 proof ethanol. Further dilutions were made in plain DMEM. The final concentrations of melatonin in the treatment medium ranged from 0.01 to 10 mM. For the vehicle control, cells were treated with absolute ethanol at a final concentration of 0.4%. After 24, 48 and 72 h of incubation, cell growth, cell viability, metabolic activity and cell cycle phase distribution were analyzed. Cell viability was measured using the standard trypan blue dye exclusion method. Fold growth was measured by counting cells electronically using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL).

Metabolic Activity: Cellular metabolic activity was measured using the Promega Cell Titer 96^{Tm} non-radioactive cell proliferation assay, following the manufacturer's protocol (Promega, Madison, WI). Briefly, triplicate cultures containing 1 x 10^4 cells in 100μl medium were seeded in a 96-well plate. Cells were incubated in a humidified 5% CO_2/95% air incubator for 24 h in the presence or absence of melatonin (at final concentrations of 0.01, 0.1, 1 and 10 mM). In the last 4h at each incubation time, MTT reagent (Promega, WI) was added. The cellular reduction of MTT by mitochondrial dehydrogenase to a blue formazon product was measured at 570 nm using a 96-well plate reader (Dynatech MR 5000, Chantilly, VA).

Cell Cycle Phase Distribution: For flow cytometry analysis, single cell suspensions were prepared by trypsinizing the cells, followed by washing in Mg^{++}/Ca^{++} free phosphate buffered saline (PBS). The cells were then fixed in paraformaldehyde at a final concentration of 5% at room temperature for 10 min. Cells were stained by a modified Krishan technique (Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol., 66, 188-195, 1975). Briefly, 1 x 10^6 cells /ml were resuspended in a hypotonic sodium citrate solution containing 50 μg/ml propidium iodide, 0.3% NP-40 and 1 mg/ml RNase-A. The cells were vortexed and stained for 30 min at room temperature in the dark. Prior to flow cytometric measurements, samples were filtered through a 37μm nylon mesh and stored at 4°C until analysis was done. All samples were analyzed with an EPICS ELITE Flow Cytometer (Coulter Cytometry, Miami, FL) using a 15mWatt air cooled argon ion laser operated at 7 amps of power at 488 nm. Histograms were analyzed for cell cycle compartments using MutiCycle-Plus Version 3.0 (Phoenix Flow Systems, San Diego, CA).

Status:

Among the many reported protective effects of melatonin, including immunomodulation during acute stress, endogenous hydroxyl radical scavenging, and inhibition of tumor growth in experimental

animal models, our laboratory has observed radioprotective effects of melatonin in normal human lymphocytes. As a preliminary study, before examining the radioprotective effect of melatonin in proliferating 293S human embryonic kidney cells, we have investigated the effect of melatonin treatment on several normal cellular processes. These include cell growth, viability, metabolic activity and cell cycle phase distribution. When cell viability was measured at the selected time points by the trypan blue dye exclusion method, no significant cell death was observed. Even after the 2 mM melatonin treatment for 72h, the viability remain at 98% (Figure 1). When the metabolic activity was determined at 24, 48 and 72 h using the colorimetric MTT assay, no significant change in metabolic activity was observed. Even with the 10 mM melatonin treatment for 72h, the metabolic activity was similar to that of control. (Figure 2) When cell cycle analysis was performed by flow cytometry, no marked difference in cell cycle distribution was observed. Melatonin at 2 mM concentration did slightly increase the percentage of SPF phase cells compared to controls after 72 h (Figure 3). This study indicated that when 293S cells are treated with concentrations of melatonin up to 2 mM, a concentration which was previously found to protect against radiation-induced chromosome aberrations in human lymphocytes (Vijayalaxmi et al, 1995), no significant alterations in the assayed cellular functions in the 293S human embryonic kidney cells occurred.

5. PERSONNEL SUPPORTED

Mohan Natarajan, Ph.D., Assistant Professor (UTHSCSA) , Co-Investigator
(70% AFOSR support, 30% UTHSCSA support)
Vijayalaxmi, Ph.D., Associate Professor (UTHSCSA), Co-Investigator
(70% time AFOSR support, 30% time UTHSCSA support)
Maria Szilagyi, Senior Research Assistant (UTHSCSA), 100% AFOSR support
Martin L. Meltz, Ph.D., Professor and CERT Director (UTHSCSA), 100% support
By UTHSCSA, with 25% time assigned to this project

6. PUBLICATIONS

VIJAYALAXMI, Mohan N, Meltz ML and Wittler MA. Proliferation and cytogenetic studies in human blood lymphocytes exposed in vitro to 2450-MHz radiofrequency radiation. International Journal of Radiation Biology. 72, 751-757, 1997.

VIJAYALAXMI, Frei MR, Dusch SJ, Guel V, Meltz ML and Jauchem J. Correction of an error in calculation in the article "Frequency of micronuclei in the peripheral blood and bone marrow of cancer-prone mice chronically exposed to 2450-MHz radiofrequency radiation". Radiation Research, 147, 495-500, 1997.

7. INTERACTIONS/TRANSITIONS

a. Participation/presentations at meetings, conferences, seminars

Mohan, N., Hunt, R., Meltz, M.L., Mohan, S. and Herman, T.S: Interleukin 1 Promoter Activation by Ionizing radiation is Mediated by Nuclear Factor-kB. Presented at the Fourth Joint Conference of the American Association for Cancer Research and the Japanese Cancer Association, February 16-21, Maui, Hawaii, 1998.

b. Consultative and Advisory Functions to other laboratories

Standards Coordinating Committee 28 (SCC-28) of IEEE

Dr. Meltz serves on Standards Coordinating Committee-28 of the IEEE. He is facilitating the computerization of the review of the existing microwave bioeffect literature, and the review itself.

Committee on Man and Radiation (COMAR) of the EMBS Society

Dr. Meltz participates actively in the meetings and deliberations of the Committee on Man and Radiation of the Engineering in Medicine and Biology Society. COMAR is actively involved in preparing documents attesting to safety and health issues relating to non-ionizing electromagnetic radiation sources found in the environment.

Cooperative Research and Development Agreement (CRDA)

Collaborative research activity continues under the CRDA between the UTHSCSA and the U.S. Air Force Research Laboratory, Brooks AFB, Texas..

Center for Environmental Radiation Toxicology (CERT)

The activities of the CERT continue. Dr. Meltz is the Director of the CERT, and Dr. Michael Murphy of the Directed Energy Division of the U.S. Air Force Research Laboratory serves on the CERT Steering Committee.

c. Transitions (Describe cases where knowledge resulting from your effort is used, or will be used, in a technology application. None.

8. NEW DISCOVERIES, INVENTIONS, OR PATENT DISCLOSURES
None

9. HONORS/AWARDS
None

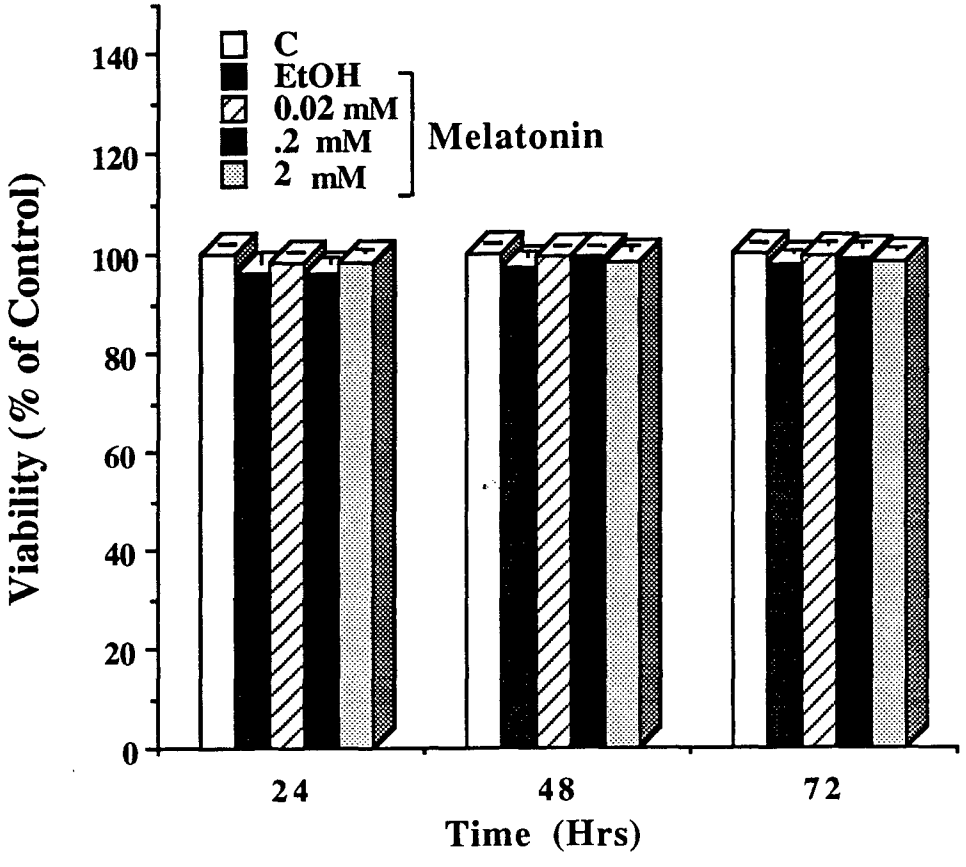

Figure 1: Effect of melatonin on cell viability. Human embryonic kidney cells (293S), seeded at a density of 1x 10^6 cells/ml, were allowed to attach overnight. Cells were then treated with 0.02, 0.2, and 2 mM of melatonin. For vehicle control, cells were treated with 0.4% absolute ethanol (the amount used in the highest concentration of melatonin). The cells were examined for viability by trypan blue dye exclusion after 24, 48 and 72 h of treatment. Each point is the arithmetic mean ± SD of two cell counts from two independent treatment flasks and is expressed as a percentage of the viability of untreated control cells.

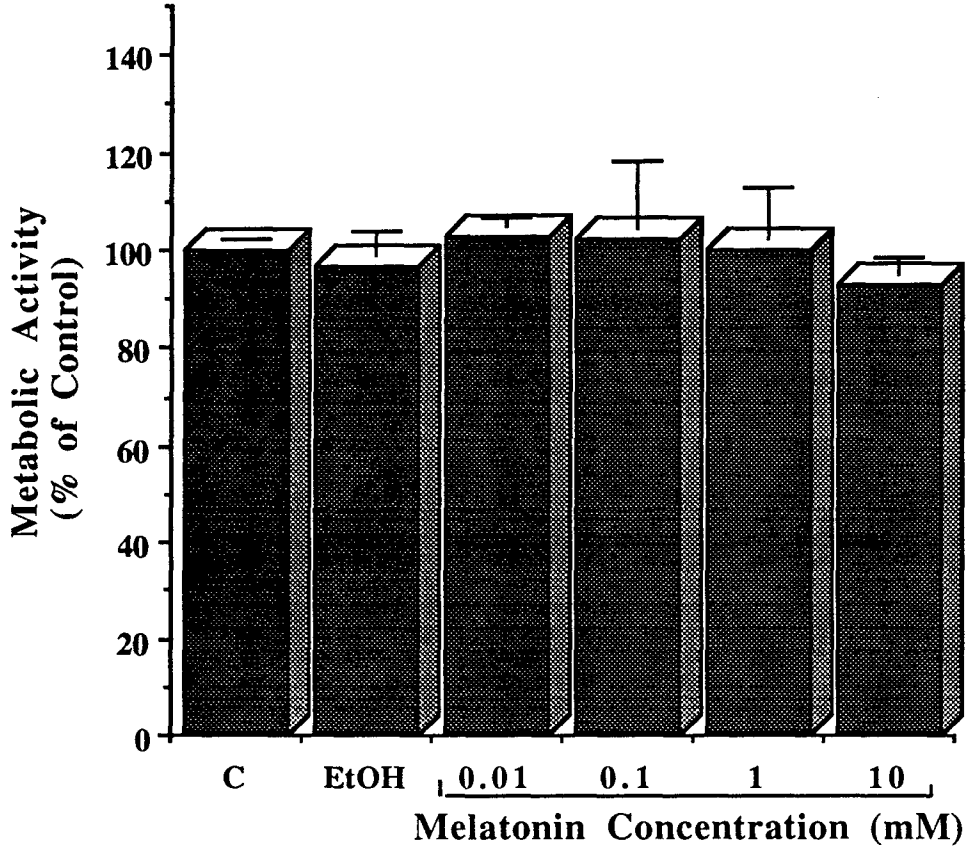

Figure 2: Effect of melatonin on cellular metabolic activity. Triplicate cultures containing 1 x 10^4 cells in 100µl medium were seaded in a 96 well plate. Melatonin at final concentrations of 0.01, 0.1, 1 and 10 mm was added and the cells incubated for 24h. For the last 4 h MTT reagent (Promega, WI) was added. The cellular reduction of MTT by mitochondrial dehydrogenase to a blue formazon product was measured at 570 nm using a 96 well plate reader. The data is the arithmetic mean ± SD of three experiments from triplicate cultures, and is expressed as a percentage of the metabolic activity of untreated control cells.

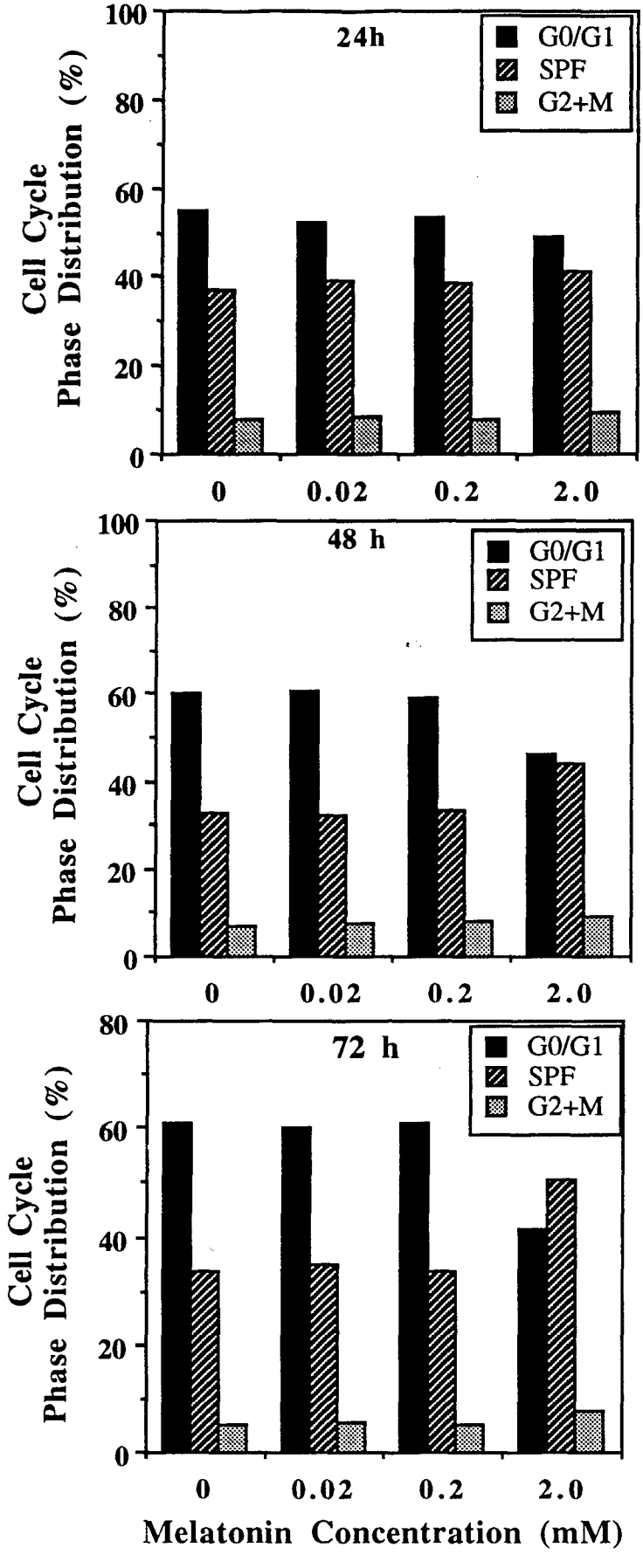

Figure 3: Cell cycle phase distribution after melatonin pre-treatment.

APPENDIX

Grant No. F49620-95-1-0037

Mechanism(s) of Molecular Responses in Mammalian Cells Exposed to Physical Agents (Alone and in Combinaation)

INT. J. RADIAT. BIOL 1997, VOL. 72, NO. 6, 751–757

Proliferation and cytogenetic studies in human blood lymphocytes exposed *in vitro* to 2450 MHz radiofrequency radiation

VIJAYALAXMI*, N. MOHAN, M. L. MELTZ and M. A. WITTLER

(Received 19 March 1997; accepted 17 July 1997)

Abstract. Aliquots of human peripheral blood collected from two healthy human volunteers were exposed *in vitro* to continuous wave 2450 MHz radiofrequency radiation (RFR), either continuously for a period of 90 min or intermittently for a total exposure period of 90 min (30 min on and 30 min off, repeated three times). Blood aliquots which were sham-exposed or exposed *in vitro* to 150 cGy gamma radiation served as controls. The continuous wave 2450 MHz RFR was generated with a net forward power of 34·5 W and transmitted from a standard gain rectangular antenna horn in a vertically downward direction. The mean power density at the position of the cells was 5·0 mW/cm^2. The mean specific absorption rate calculated by Finite Difference Time Domain analysis was 12·46 W/kg. Immediately after exposure, lymphocytes were cultured for 48 and 72 h to determine the incidence of chromosomal aberrations and micronuclei, respectively. Proliferation indices were also recorded. There were no significant differences between RFR-exposed and sham-exposed lymphocytes with respect to: (a) mitotic indices; (b) incidence of cells showing chromosome damage; (c) exchange aberrations; (d) acentric fragments; (e) binucleate lymphocytes, and (f) micronuclei, for either the continuous or intermittent RFR exposures. In contrast, the response of positive control cells exposed to 150 cGy gamma radiation was significantly different from RFR-exposed and sham-exposed lymphocytes. Thus, there is no evidence for an effect on mitogen-stimulated proliferation kinetics or for excess genotoxicity within 72 h in human blood lymphocytes exposed *in vitro* to 2450 MHz RFR.

1. Introduction

Non-ionizing radiofrequency radiation (RFR) in the microwave range is having a significant and positive impact on modern society through a multitude of uses including communications and broadcasting; various types of radar; industrial uses such as welding metals, heating and sealing plastics; medical uses such as diathermy and heating to kill cancerous cells; and domestic uses such as cooking, portable telephones and cellular devices. There has been a growing concern among the public regarding the potential human health hazards of exposure to radiofrequency radiation emitted during such uses. Although the primary effect of the energy from RFR on living tissues appear to be due to an increase in temperature (Stuchley 1988), a number of biological effects have been reported without a measured increase in temperature (Hitchcock and Patterson 1994). While some human epidemiological studies suggested an association of possible RFR exposures with certain kinds of cancer, Hitchcock and Patterson (1994) concluded that 'the data do not support the finding that exposure to RF fields is a causal agent for any type of cancer'. Chronic animal studies which were designed to explore the question of carcinogenesis used normal and tumour-prone mice exposed to 2450 MHz RFR, and the data provided positive (Szmigielski *et al.* 1982), questionable (Chou *et al.* 1992), and negative results (Santini *et al.* 1988, Wu *et al.* 1994, Frei *et al.* 1997).

Since damage to the genetic apparatus would imply the possibility of an initiating event for carcinogenesis, the genotoxic potential of RFR has been examined in several *in vivo* and *in vitro* studies using both rodent and human cells. While the majority of the studies, where exposure to 2450 MHz was examined, indicated no significant differences (Leonard *et al.* 1983, Beechey *et al.* 1986, Lloyd *et al.* 1986, Ciaravino *et al.* 1987, Saunders *et al.* 1988, Meltz *et al.* 1989, Kerbacher *et al.* 1990, Meltz *et al.* 1990, Ciaravino *et al.* 1991, Vijayalaxmi *et al.* 1997), a recent study reported increased incidence of dicentric chromosomes, acentric fragments and micronuclei in human blood lymphocytes (Maes *et al.* 1993). In view of: (i) the continuing debate about possible adverse health effects of RFR exposures; (ii) the potential implication and importance of the positive findings of Maes *et al.* (1993); and (iii) the negative observations made in earlier studies using other cell types, the effects of *in vitro* exposure to continuous wave 2450 MHz RFR on mitogenic stimulation, proliferation kinetics and the extent of cytogenetic damage in human blood lymphocytes have been examined; the 2450 MHz RFR was chosen because it is one frequency for which there is considerable negative experimental data, but for which a few positive results have been reported. The exposures were either continuous for a period of 90 min, or

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intermittent for a total period of 90 min (30 min on and 30 min off, repeated three times). The selection of 30 min on and 30 min off for intermittent RFR exposure was admittedly arbitrary; it was intended to investigate a possible 'stressor effect' of turning the RFR on and off repeatedly, and not to mimic any current technology use (which in fact is highly variable). The cytogenetic effect of such an intermittent exposure condition has not been investigated in any cell type.

2. Materials and methods

2.1. *Blood collection*

The protocol approved by the Institutional Review Board of The University of Texas Health Science Center at San Antonio for handling the blood samples collected from human volunteers was followed. The blood donors were two healthy, non-smoking males of 35 and 55 years of age. From each donor, two blood samples were collected in heparinized vacutainer tubes, 4 days apart. The first blood sample from each donor was used for the continuous exposure study, and the second sample for the intermittent exposure study. At each collection time, 6 ml aliquots of whole blood from each donor were distributed into nine separate T-25 tissue culture flasks. Three flasks were exposed to 2450 MHz RFR, three flasks were sham-exposed, and three flasks were exposed *in vitro* to 150 cGy gamma radiation (positive controls).

2.2. *RFR exposure*

The RFR exposure facility is an anechoic room measuring 18·0 × 9·0 × 9·0 ft. The temperature of the room was maintained at 37 ± 1°C. The RFR was generated using a custom designed MPD Dual Frequency Transmitter (Micon Inc., Owensboro, KY, USA). The net forward power at 2450 MHz was 34.5 W. The continuous wave 2450 MHz RFR was transmitted from a Narda (Hauppauge, NY, USA) standard gain antenna horn (35·5 × 25·5 cm) in a vertically downward direction.

Three flasks, each containing 6 ml of whole blood, and a fourth flask containing only 6 ml of RPMI 1640 tissue culture medium (for temperature monitoring; Mediatech, Washington, DC) were placed in four separate impressions cut into the underside of four plugs fitting into the top of a pressed-foam incubator box measuring 36·0 × 27·0 × 13·5 cm. The incubator box was placed directly under the antenna horn. The position of the cells at the bottom of the culture flasks were at 1·75 m from the opening of the antenna horn. The beginning of the far field was calculated to be at 2·0 m (calculated using $d = 2D^2/\lambda$; d is the distance at which the far field begins; D is the longest axis of the antenna horn). Before the RFR exposure began, the temperature in the flasks was equilibrated at 37°C. This was accomplished by flowing warm air through tubing immersed in a water bath, and directing it into the incubator box. The water bath temperature was adjusted (>37°C) to obtain a measured temperature of 37°C in the medium in the monitoring flask. The temperature was continuously monitored before and during the RFR exposure using a BSD (Salt Lake City, UT, USA) Medical Corporation Model 200 Thermometry System; a non-RFR-interactive Vitek probe was inserted down through a hole drilled in a foam plug and into the bottom of the medium in the temperature monitoring flask. During the RFR exposure, the incubator box was gently rocked side-to-side to gently agitate the cells. The mean power density measured at the distance of the cells from the antenna horn was 5·0 mW/cm^2 (variation of mean ± 7% for measurements made at four flasks position); this was determined using a calibrated Narda (Hauppauge, NY, USA) Model 8616 electromagnetic radiation monitor and a Model 8612D E-field probe. The power density is in the range of the level of existing guidelines for RFR exposures. This power density allowed for an increase in the temperature during RFR exposure, but this increase was restricted to below 39°C, where hyperthermic effects might become an issue. The SAR was not determined at the time of experimentation. Instead, the SAR was calculated using Finite Difference Time Domain (FDTD) analysis (by Dr Arthur W. Guy, personal communication) with special attention given to the differences observed in the miniscus height at different positions around the T-25 flask. The miniscus heights were measured by John McDougal and Dr C. K. Chou, City of Hope Medical Center (personal communication), with RPMI 1640 medium containing 10% serum in the flask to represent the same volume of blood. At the bottom of the flask, using ϵ (58·12) and σ (2·545) for whole blood in the FDTD calculations, the mean SAR was 12·46 (± 0·1 standard error) W/kg. The SAR values varied at different positions, ranging from $1{\cdot}8 \times 10^{-7}$ to 47·05 W/kg. Based on the FDTD analysis, more than 75% of the cells were exposed to SARs greater than 1.72 W/kg, and more than 50% of the cells were exposed to SARs greater than 6.53 W/kg. The 2450 MHz RFR exposure was conducted either continuously for a period of 90 min, or intermittently (30 min on and 30 min off, repeated three times) for a total exposure time of 90 min.

2.3. *Sham-exposure*

Three flasks, each containing 6 ml of whole blood and a fourth flask containing only 6 ml of tissue culture medium (for temperature monitoring) were fixed into a second identical 'incubator box'. This box was placed at the far end of the anechoic room away from the RFR field, and was also gently rocked. The flasks were treated in the same way as the RFR exposed flasks.

2.4. *Positive controls*

Three flasks, each containing 6 ml of whole blood, were placed in the anechoic room next to the sham-exposure incubator box during the RFR exposure; these flasks remained stationary. At the end of the RFR exposure, these positive control flasks were taken out of the anechoic room and immediately exposed *in vitro* to an acute dose of 150 cGy gamma radiation at room temperature. The radiation was delivered using a Cs-137 source (GammaCell-40, Atomic Energy of Canada Ltd.) at a dose rate of 108·7 cGy/min.

2.5. *Chromosomal aberrations*

Immediately after RFR-, sham- and gamma radiation-exposures, separate cultures were set up by mixing 1 ml aliquots of whole blood from each flask with 9 ml of complete RPMI 1640 culture medium (Mediatech, Washington, DC) containing 15% foetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 1% phytohemagglutinin (PHA: Gibco, Grand Island, NY, USA), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine (Mediatech) and 25 μM bromodeoxyuridine (Sigma, St. Louis, MO, USA). All cultures were incubated for 48 h at 37 ± 1°C in a humidified atmosphere of 5% CO_2 and 95% air. During the last 2 h of incubation, colcemid (Gibco) was added to all cultures at a final concentration of 0·1 μg/ml. At the end of the 48 h, the lymphocytes were collected by centrifugation, treated with 75 mM potassium chloride for 6–8 min, and fixed in a 3:1 methanol:acetic acid mixture. Fixed cells were dropped onto clean microscope slides and air dried. Fluorescence-plus-Giemsa stained (Perry and Wolff 1975) slides were coded by an individual other than the scorer, and then evaluated for chromosome aberrations. For each donor, from each culture, 2000 consecutive cells were examined for mitotic index, and 200 lymphocytes in their first mitotic division (defined by the absence of harlequin staining) were examined for chromosomal aberrations (Vijayalaxmi *et al.* 1995a).

2.6. *Micronuclei*

Separate cultures were set up by mixing 1 ml aliquots of whole blood from each flask with 9 ml of the above described complete culture medium, but without added bromodeoxyuridine. These cultures were incubated for 72 h at 37 ± 1°C in a humidified atmosphere of 5% CO_2 and 95% air. Cytochalasin B (4 μg/ml; Sigma, St. Louis, MO, USA) was added to all cultures at 44 h to arrest the dividing cells in cytokinesis. At the end of the 72 h, lymphocytes were collected by centrifugation, treated with 0·8% sodium citrate for 3–5 min, and fixed in a 5:1 methanol acetic acid mixture. Fixed cells were dropped gently onto clean microscope slides, air dried and stained with 4% Giemsa (Sigma). All slides were coded by an individual other than the scorer and then evaluated at 1000 × magnification (Vijayalaxmi *et al.* 1995b). For each donor, from each culture, 2000 consecutive cells were examined for the incidence of binucleate (BN) cells, and 2000 BN cells were examined to record the frequency of cells with one (C1MN), two (C2MN) or three (C3MN) micronuclei. The number of BN cells containing micronuclei was assessed as (C1MN) + (C2MN) + (C3MN). The total number of micronuclei observed were derived from (1 × C1MN) + (2 × C2MN) + (3 × C3MN).

2.7. *Statistical analysis*

All data were analysed using the Students *t*-test. In addition, the data were subjected to the analysis of variance (ANOVA) test using donor as a random effect, and treatment (sham-, RFR- and gamma radiation-exposed) and time (continuous versus intermittent) as fixed factors; all interactions of these factors were considered. The log transformation (Zar 1974) assured homogeneous variances for fair comparison of the means much better than the raw data, and this was used to obtain *p* values. Those less than 0·05 were considered statistically significant. This multiplicative model of the raw data or additive model for the log scale was applied to all seven response variables. Least squares means were compared with *t*-tests using the appropriate error variability from the analysis of variance model.

3. Results

The overall cytogenetic responses of the lymphocytes from the two donors to different exposure conditions were similar, although the absolute values were slightly different. The percent mitotic indices, the incidence of cells showing chromosomal damage, the numbers of exchange aberrations and excess acentric fragments in the lymphocytes exposed to the

Table 1. The mitotic indices, the incidence of cells with chromosome damage, numbers of exchange aberrations and acentric fragments induced *in vitro* by 2450 MHz radiofrequency radiation (RFR) in cultured human blood lymphocytes.

Treatment	Percent mitotic index	Cells with chromosome damage in 200 metaphases	Exchange aberrations in 200 metaphases	Excess fragments in 200 metaphases
RFR–Continuous Exposure for 90 min				
Blood donor 1:				
Control	4·7 ± 0·69	2·0 ± 0·00	0·0 ± 0·00	2·0 ± 0·00
2450 MHz RFR	5·1 ± 0·17	3·0 ± 1·00	0·3 ± 0·58	2·7 ± 1·15
150 cGy gamma radiation	4·4 ± 0·10	64·7 ± 5·51	54·7 ± 4·04	31·7 ± 2·52
Blood donor 2:				
Control	4·0 ± 0·15	3·3 ± 0·58	0·0 ± 0·00	3·3 ± 0·58
2450 MHz RFR	4·3 ± 0·15	4·3 ± 0·58	0·3 ± 0·58	4·0 ± 1·00
150 cGy gamma radiation	3·3 ± 0·50	66·3 ± 1·53	48·0 ± 7·00	36·3 ± 8·33
RFR–Intermittent Exposure (30 min ON, 30 min OFF, 3 ×)				
Blood donor 1:				
Control	4·9 ± 0·35	2·7 ± 1·15	0·0 ± 0·00	2·7 ± 1·15
2450 MHz RFR	5·2 ± 0·06	4·0 ± 1·00	0·3 ± 0·58	3·7 ± 0·58
150 cGy gamma radiation	4·2 ± 0·31	61·3 ± 2·52	47·0 ± 3·00	43·3 ± 11·1
Blood donor 2:				
Control	4·4 ± 0·21	3·3 ± 0·58	0·0 ± 0·00	3·3 ± 0·58
2450 MHz RFR	4·9 ± 0·46	4·0 ± 1·00	0·3 ± 0·58	3·7 ± 1·15
150 cGy gamma radiation	4·0 ± 0·36	65·0 ± 11·7	49·7 ± 7·09	42·0 ± 6·08

The data are mean ± standard deviation.

2450 MHz RFR (continuous and intermittent) were not significantly different from those in sham-exposed cells (Table 1). Similarly, the percent BN cells, the incidence of BN cells with one, two, or three micronuclei, and the total numbers of micronuclei in the RFR-exposed lymphocytes were not significantly different from those in the sham-exposed cells (Table 2). In contrast, the cytogenetic responses of the cells which were exposed *in vitro* to an acute dose of 150 cGy gamma radiation were significantly different from both the RFR-exposed and the sham-exposed lymphocytes ($p > 0{\cdot}001$). When the data between continuous and intermittent RFR exposures were compared, there were no significant differences in any of the cytogenetic parameters investigated. Also, the overall conclusions drawn were not different when the data were subjected to either the Students *t*-test or ANOVA method.

In the continuous exposure experiment, the temperatures at the beginning and at the end of the 90 min exposure were 36·9°C and 38·4°C, respectively (a rise of 1·5°C). In the intermittent exposure experiment, the temperatures at the beginning of the first, second and third RFR exposures were 36·8°C, 37·1°C and 37·5°C, respectively; the temperatures at the end of the first, second and third RFR exposures were 37·7°C, 37·9°C and 38·1°C, respectively.

4. Discussion

This seems to be the first study where the cytogenetic response of human blood lymphocytes exposed intermittently *in vitro* to 2450 MHz RFR was compared with that in cells exposed continuously for 90 min.

The response of the cells to PHA-stimulation, as indicated by the mitotic indices, and the proliferation rate at which the lymphocytes reached their second mitosis, as indicated by the incidence of BN cells, were not significantly different between RFR-exposed (continuous and intermittent) and sham-exposed cells. These observations are in accordance with the results of Roberts *et al.* (1983). These investigators exposed human mononuclear leukocytes to continuous wave 2450 MHz RFR for 2 h (SARs of 0·5–4·0 mW/g), and then cultured the cells for 5–7 days with and without PHA-stimulation. Determinations of lymphocyte viability, and unstimulated or stimulated DNA, RNA, total protein and interferon synthesis, did not indicate any significant differences between RFR-exposed, sham-exposed and unexposed control cells. These results are in contrast with the observations reported by Cleary *et al.* (1990). The latter investigators exposed human whole blood *in vitro* for 2 h to continuous wave 2450 MHz RFR at 37·0 ± 0·1°C (SARs of 5–50 W/kg), separated the lymphocytes and then cultured the cells for 3 days with and without mitogen (PHA) stimulation. During the final 6 h of the culture period, the cells were pulse labelled with ^{3}H-thymidine (^{3}H-TdR) to determine its incorporation into cellular DNA. Their data indicated that relative to mitogen-stimulated sham-exposed controls,

Table 2. The percentages of binucleate cells (BN), and the incidence of BN cells with 1 (C1BN), 2 (C2BN) or 3 (C3BN) micronuclei (MN), BN cells with MN, and total MN induced *in vitro* by 2450 MHz radiofrequency radiation (RFR) in cultured human blood lymphocytes.

Treatment	Percent BN cells	Total BN cells with 1 MN (C1MN)	Total BN cells with 2 MN (C2MN)	Total BN cells with 3 MN (C3MN)	BN cells with MN in 2000 BN cells (@)	Total MN in 2000 BN cells (@@)
RFR–Continuous Exposure for 90 min						
Blood donor 1:						
Control	50 ± 1·53	61	0	0	20 ± 0·58	20 ± 0·58
245 MHz RFR	52 ± 1·73	65	1	0	22 ± 1·73	22 ± 2·08
1·5 Gy gamma radiation	46 ± 1·15	796	68	22	295 ± 20·0	333 ± 25·8
Blood donor 2:						
Control	49 ± 0·58	67	1	0	23 ± 0·58	23 ± 1·00
2450 MHz RFR	50 ± 1·53	74	0	0	25 ± 1·53	25 ± 1·53
1·5 Gy gamma radiation	43 ± 1·53	841	67	16	308 ± 34·0	341 ± 32·5
RFR-Intermittent Exposure (30 min ON and 30 min OFF, 3 ×)						
Blood donor 1:						
Control	51 ± 1·53	73	2	0	25 ± 1·73	26 ± 2·52
2450 MHz RFR	50 ± 1·53	84	0	0	28 ± 4·36	28 ± 4·36
1·5 Gy gamma radiation	46 ± 1·73	799	42	10	284 ± 30·6	304 ± 30·0
Blood donor 2:						
Control	48 ± 1·73	71	0	0	24 ± 1·73	24 ± 1·53
2450 MHz RFR	49 ± 3·00	81	1	0	27 ± 0·58	28 ± 1·15
1·5 Gy gamma radiation	43 ± 1·00	737	49	12	266 ± 14·4	290 ± 15·2

The data are mean ± standard deviation.
(@) : (C1MN) + C2MN) + (C3MN).
(@@): (1 × C1MN) + (2 × C2MN) + (3 × C3MN).

mitogen-stimulated RFR-exposed lymphocytes exhibited RFR-intensity-dependent alterations in ^{3}H-TdR uptakes, with a significant decrease at an SAR of 50 W/kg, and increases at SARs of 25 or 39·5 W/kg. While the authors did not present any data, they stated that 'in the absence of mitogen, there was a statistically significant (142%, $p < 0·03$) increase of uptakes' and 'at intensities of radiation that induced proliferation, this response was consistently enhanced in the absence of mitogenic activation by PHA'.

The energy of the RFR is too low to cause direct breakage of the covalent bonds in DNA molecules. Sagripanti and Swicord (1986) did, however, report that exposure of an aqueous solution of pUc8.c2 plasmid DNA (5480 base pairs long) to 2·55 GHz RFR at 20°C for 20 min (SARs of 21–85 mW/g) resulted in an SAR-dependent decrease in supercoiled DNA with an accompanying increase of relaxed and linear DNA, suggesting the formation of single and double strand breaks. These observations are inconsistent with the failure of Gabriel *et al.* (1990) to reproduce the absorption of 1–10 GHz RFR energy by the same plasmid DNA. More recently, rearranged DNA fragment patterns were reported in DNA samples isolated from the testis and brain of mice exposed to 2450 MHz RFR (SAR of 1·18 W/kg), 2 h/day for 120, 150, 200 days (Sarkar *et al.* 1994). Also, Lai and Singh (1995, 1996), using the microgel electrophoresis assay, reported an increase in both single and double strand breaks in the DNA of freshly isolated brain cells of rats at 4 h after they were exposed to 2 h of 2450 MHz (SARs of 0·6 and 1·2 W/kg). These authors: (i) suggested a possibility that RFR-induced low levels of genetic damage was not detectable by the assay methods used in several published negative reports, indicating that the comet assay was highly sensitive and able to detect such low levels of DNA damage; and (ii) speculated that their observed effects could be due to a direct action of RFR on DNA molecules and/or impairment of DNA damage repair mechanisms in brain cells. If an impairment of DNA repair mechanism is involved in RFR-induced genetic damage, one would expect that the unrepaired and/or misrepaired DNA damage should result in enchanced frequencies of chromosomal aberrations, micronuclei or mutations; the observations made in the present study and several other negative reports did not substantiate such speculation.

While the study was limited to blood samples examined from two individuals, differences could arise as a consequence of genetic variability. Previous unrelated studies using the blood from the same two donors indicated that the response of their cells was

similar for gamma radiation-induced chromosomal aberrations and micronuclei. In the absence of a positive outcome (i.e. enhanced chromosomal aberrations and/or micronuclei following RFR exposure) in the blood sample of at least one of the individuals, and in view of an overwhelming weight of evidence that radiofrequency radiation exposures (without heating) do not cause genetic damage, it is difficult to justify more extensive investigations and examination of cells from more blood donors.

Recently, Maes *et al.* (1993) examined the effect of *in vitro* exposure of human peripheral blood lymphocytes to 2450 MHz RFR for 30–120 min at a constant temperature of $36{\cdot}1 \pm 0{\cdot}1°C$ (SAR of 75 W/kg). While their observations did not indicate any remarkable influence of RFR on cell cycle kinetics or sister chromatid exchange frequencies, they did, report a significant increase in chromosomal aberrations (including dicentric chromosomes and acentric fragments) and micronuclei in RFR exposed lymphocytes. These data are difficult to validate because of the questionable RFR-dosimetry and temperature measurements described in the paper. These authors: (i) used a metallic thermister microprobe, passed through a hypodermic syringe needle, which was placed axially halfway down in the blood exposed to RFR; the tip of the microprobe extending beyond the syringe needle into the blood could still be interactive with the RFR; (ii) immersed a hypodermic needle in the blood to avoid self-heating and microwave pick-up by the thermister probe which could itself act as an antenna, and could heat up and damage any cells which came in contact with it; (iii) calculated the SAR based on a separate experiment which did not involve RFR exposure, but fed a DC current through an immersed electric resistor which was moved in the medium to homogenize the temperature distribution—a procedure that was not followed during the RFR-exposure of the blood sample; and (iv) assumed uniform SAR across and at various depths of the tube containing the blood, which may not necessarily be the case. Under the 2450 MHz RFR-exposure conditions used in the present study, the results indicate no significant differences between RFR-exposed and sham-exposed lymphocytes with respect to proliferation kinetics, chromosomal aberrations and micronuclei in human blood lymphocytes. Also, no significant differences in cytogenetic responses between continuous and intermittent RFR exposures were observed.

Acknowledgements

This work was supported by the United States Air Force Office of Scientific Research grant No. F49620–95–1-0337. We thank Dr Thomas J. Prihoda for statistical analysis of the data.

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RADIATION RESEARCH 147, 495–500 (1997)
0033-7587/97 $5.00

SHORT COMMUNICATION

Frequency of Micronuclei in the Peripheral Blood and Bone Marrow of Cancer-Prone Mice Chronically Exposed to 2450 MHz Radiofrequency Radiation

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Vijayalaxmi, Frei, M. R., Dusch, S. J., Guel, V., Meltz, M. L. and Jauchem, J. R. Frequency of Micronuclei in the Peripheral Blood and Bone Marrow of Cancer-Prone Mice Chronically Exposed to 2450 MHz Radiofrequency Radiation. *Radiat. Res.* 147, 495–500 (1997).

C3H/HeJ mice, which are prone to mammary tumors, were exposed for 20 h/day, 7 days/week, over 18 months to continuous-wave 2450 MHz radiofrequency (RF) radiation in circularly polarized wave guides at a whole-body average specific absorption rate of 1.0 W/kg. Sham-exposed mice were used as controls. The positive controls were the sentinel mice treated with mitomycin C during the last 24 h before necropsy. At the end of the 18 months, all mice were necropsied. Peripheral blood and bone marrow smears were examined for the extent of genotoxicity as indicated by the presence of micronuclei in polychromatic erythrocytes (PCEs). The results indicate that the incidence of micronuclei/1,000 PCEs was not significantly different between groups exposed to RF radiation (62 mice) and sham-exposed groups (58 mice), and the mean frequencies were 4.5 ± 1.23 and 4.0 ± 1.12 in peripheral blood and 6.1 ± 1.78 and 5.7 ± 1.60 in bone marrow, respectively. In contrast, the positive controls (7 mice) showed a significantly elevated incidence of micronuclei/1,000 PCEs in peripheral blood and bone marrow, and the mean frequencies were 50.9 ± 6.18 and 55.2 ± 4.65, respectively. When the animals with mammary tumors were considered separately, there were no significant differences in the incidence of micronuclei/1,000 PCEs between the group exposed to RF radiation (12 mice) and the sham-exposed group (8 mice), and the mean frequencies were 4.6 ± 1.03 and 4.1 ± 0.89 in peripheral blood and 6.1 ± 1.76 and 5.5 ± 1.51 in bone marrow, respectively. Thus there was no evidence for genotoxicity in mice prone to mammary tumors that were exposed chronically to 2450 MHz RF radiation compared with sham-exposed controls.

INTRODUCTION

Nonionizing radiofrequency (RF) radiation in the frequency range of 300 MHz to 300 GHz (microwaves) has long been used in radar, space research, telecommunications, industrial processing and medicine. A large increase in commercial use and in the number of people who are potentially exposed to RF radiation occurred with the introduction of household microwave ovens, which operate predominantly at 2450 MHz. With the introduction of cellular telephones, even more public attention has been drawn to the possible bioeffects and health hazards of exposure to RF radiation. The most obvious bioeffects of microwaves reported are associated with heating generated by RF radiation that occurs when the specific absorption rate (SAR) is high. Although the human body dissipates excess thermal energy very efficiently, significant heat-related bioeffects involving major organ systems have been reported in association with specific RF-radiation frequencies, incident power densities, and AM, FM or pulsed modulations (*1–3*). Some human epidemiological studies have suggested carcinogenic, co-carcinogenic or tumor-promoting properties of RF radiation (*4–6*). Results from studies of long-term 2450 MHz RF-radiation exposure involving normal and tumor-prone mice have provided positive (*7*), questionable (*8*) and negative results (*9–11*). In a series of *in vitro* studies using C3H 10T1/2 cells, Balcer-Kubiczek and Harrison (*12–14*) reported that exposure to 2450 MHz RF radiation alone (for 24 h) had no effect on cell survival or induction of neoplastic transformation, while enhancement of transformation was significant when RF radiation was combined with the tumor-promoting agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and additive with doses of X rays given as a co-carcinogen. In their experiments, the number

of dishes seeded for the transformation assay, even within one experiment, varied considerably between treatment conditions, raising a question about the statistical validity of the data. Nonetheless, in view of the potential importance of the reported findings, these studies need independent confirmation. Several researchers have examined the genotoxic potential of 2450 MHz RF radiation in *in vivo* experiments and in *in vitro* investigations using rodent and human cells. While the majority have reported no significant differences (*15–23*), some did indicate positive effects, including increased incidence of dicentric chromosomes, acentric fragments and micronuclei (*24*), and rearranged DNA fragment patterns (*25*). More recently, Lai and Singh (*26*) used the comet assay (gel electrophoresis) to detect DNA single-strand breaks in brain cells of rats exposed (whole-body) for 2 h to pulsed-wave (PW) or continuous-wave (CW) 2450 MHz RF radiation. They reported either no increase (PW) or a significant increase (CW) in the length of DNA migration immediately after the 2-h exposure to RF radiation ended, and a significant increase in the length of DNA migration for both PW and CW at 4 h after exposure. In their paper: (a) No valid reason or plausible mechanism was proposed to explain the contrast between the results obtained immediately compared to 4 h after the PW exposure compared with the CW exposure. (b) The data obtained were inconsistent. In a PW experiment, for a 1.2-W/kg exposure, the length of DNA migration observed 4 h after exposure was considerably greater than that measured immediately after exposure, while the two values were similar after the 0.6-W/kg exposure. In the CW experiment, for 1.2 W/kg, the values were again similar between 0 and 4 h after exposure. There has been no known DNA repair mechanism which will explain these data. (c) The lengths of migration of DNA observed from brain cells of sham-exposed rats appear to be greater than reported for any other biological system by any other laboratory using the comet assay. (d) No positive controls, such as rats exposed to ionizing radiation, were included in the study. (e) Many details were not described adequately: These include the variation in the length of migration of DNA among the 50 cells examined from each rat, a statement of the number of rats exposed to RF radiation at each time, and whether a manual or computer-based image analysis system was used for microscopic evaluation of the slides.

The rodent micronucleus assay has been applied widely as an *in vivo* assay for detecting genotoxic agents and has become a standard mutagenicity assay used in regulatory testing in several countries (*27–30*). Micronuclei arise from acentric chromosome fragments or whole chromosomes that are not incorporated into daughter nuclei at the time of cell division. The assay therefore detects both clastogens and agents that affect the spindle apparatus leading to numerical chromosome aberrations. The objective of this study was to assess the extent of cytogenetic damage in peripheral blood and bone marrow cells of cancer-prone mice that were exposed chronically to 2450 MHz RF radiation. This study was part of a larger investigation designed to determine whether or not chronic exposure of C3H/HeJ mice, which are prone to mammary tumors, to 2450 MHz RF radiation promotes an earlier onset, a greater incidence or a faster growth rate of mammary tumors; these results will be presented in a separate paper. This paper describes the extent of cytogenetic damage, as assessed by the micronucleus assay.

MATERIALS AND METHODS

A protocol approved by the U.S. Air Force Armstrong Laboratory and the Trinity University Animal Care and Use Committees was followed. The Standard Operating Procedures were compatible with the requirements of the U.S. National Toxicology Program.

RF Radiation Exposure and Dosimetry

A modified version of the circularly polarized wave-guide system described by Guy *et al.*[1] was used to expose the mice to continuous-wave 2450 MHz RF radiation. The cages, originally designed to house a single rat, were modified to house two mice, allowing both mice free access to food and water. The exposure was for 20 h/day, 7 days/week, over 18 months. The signal was generated by a Hewlett Packard Signal Generator (Model 8616A) and amplified by an MCL 1-kW S-Band amplifier (Model 10704). The RF radiation power level was monitored continuously at the output terminal of the amplifier with a Boonton RF Powermeter (Model 4300) connected to a Zenith 386 computer system via an IEEE 488 bus. The software was designed to acquire and store data on RF-radiation exposure (transmitter output; forward, reflected and absorbed power). On a rotating basis, the RF-radiation output to individual, active wave guides was also recorded: each wave guide was monitored 1 day every 50 days over the entire exposure period. The SAR was determined by two different methods, one based on differential power measurement[1] and a second using a calorimetric method.[2] The average SAR value was 1.0 W/kg for two medium-sized mice per wave guide. Exposure of mice to 2450 MHz RF radiation at an SAR of 1.0 W/kg has not been shown to result in a significant change in core temperature (*31*). The transmitter was de-energized daily for 4 h (0800–1200 h) for routine animal husbandry.

Mice and Maintenance

Three- to 4-week-old female C3H/HeJ mice possessing a milk-borne virus that induces mammary gland tumors were obtained from the Jackson Laboratory, Bar Harbor, ME. After a 10-day quarantine period, 200 healthy mice were divided randomly into two groups of 100 each, anesthetized with metofane and toe-clipped for permanent identification. One group was exposed to 2450 MHz RF radiation and the other group was sham-exposed to serve as controls. An extra 25 mice were maintained as sentinel animals and used for periodic examination of health status.

All mice were kept in the same room: the temperature was maintained at 24 ± 1°C, with 50 ± 5% relative humidity, and an air-flow rate of 18 exchanges/hour. A time-controlled system provided cycles of 0600–1800 h light and 1800–0600 h dark. Two mice belonging to the same group were housed in one cage (143 cm^2 floor area/mouse) with access *ad libitum* to Purina rodent chow and water from electrically decoupled bottles that prevented conduction currents between the mouse and sipper

[1] A. W. Guy, C. K. Chou, R. B. Johnson and L. L. Kunz, Effects of long-term, low-level radiofrequency radiation exposure on rats. Design, facilities and procedures. USAF School Aerospace Medicine Technical Report 86-17, Brooks Air Force Base, TX, 1983.

[2] J. M. Padilla and R. R. Bixby, Using Dewar-flask calorimetry and rectal temperatures to determine the specific absorption rates of small rodents. USAF School Aerospace Medicine Technical Report 86-3, Brooks Air Force Base, TX 1987.

TABLE I
Micronucleus Frequencies in the Peripheral Blood and Bone Marrow of Cancer-Prone Mice Exposed Chronically (18 Months) to 2450 MHz Radiofrequency Radiation

Group	Number of mice studied	Total erythrocytes examined[a]	Group mean percentage PCEs (± SD)	Individual mouse data: micronuclei/2.000 PCEs								Group mean micronuclei/1.000 PCEs (± SD)
Peripheral blood												
Exposed to 2450 MHz RF radiation	62	62.000	2.8 (0.69)	8	9	9	12	10	10	9	10	4.5 (1.23)
				9	9	8	11	9	7	10	8	
				11	8	10	10	7	10	9	8	
				9	8	9	9	7	8	9	10	
				7	10	9	10	8	10	7	9	
				10	11	7	10	7	8	9	8	
				11	8	11	8	7	9	9	8	
				10	10	10	9	8	10			
Sham-exposed control	58	58.000	3.1 (0.71)	9	7	8	12	10	8	9	8	4.0 (1.12)
				7	9	7	8	9	10	8	9	
				8	7	7	8	9	7	8	8	
				9	8	9	7	9	6	8	9	
				6	8	7	6	8	9	7	8	
				9	8	7	9	10	7	9	8	
				8	6	8	7	8	8	7	8	
				8	7							
MMC (1 mg/kg body weight)	7	7.000	2.7 (0.68)	99	98	107	95	113	99	101		50.9 (6.18)
Bone marrow												
Exposed to 2450 MHz RF radiation	62	12.400	51.5 (3.80)	9	12	10	13	12	14	13	14	6.1 (1.78)
				12	12	13	14	14	11	12	12	
				10	14	10	13	13	14	9	14	
				14	13	11	10	14	10	11	14	
				10	13	10	11	12	15	11	10	
				13	12	9	13	12	14	9	13	
				13	9	12	14	12	11	15	11	
				14	15	12	11	9	15			
Sham-exposed control	58	11.600	52.1 (3.32)	12	9	11	14	12	11	12	10	5.7 (1.60)
				11	12	11	12	11	10	13	12	
				9	12	10	9	11	8	10	12	
				10	13	9	11	14	11	10	9	
				13	10	14	12	10	11	9	13	
				12	12	10	13	13	12	9	14	
				9	13	12	13	10	14	11	10	
				12	14							
MMC (1 mg/kg body weight)	7	1.400	46.4 (3.59)	115	112	106	105	113	116	106		55.2 (4.65)

[a]For each mouse, 1.000 consecutive erythrocytes in the peripheral blood and 200 consecutive erythrocytes in the bone marrow were examined.

tube. The cages were Plexiglas (virtually transparent to RF radiation at 2450 MHz), which permitted twice-daily visual inspection of the mice (at ~0800 h and at ~1400 h). Each week, all mice were weighed, examined and palpated for tumors.

Positive Controls

Mitomycin C (MMC), a known clastogen which has been shown to induce micronuclei (*32*), was used as a positive control substance. Seven of the 25 sentinel mice survived through the full 18-month study period; they were injected intraperitoneially with MMC (1 mg/kg body weight) and sacrificed 24 h later. From each mouse, peripheral blood and bone marrow smears were made as described below.

Peripheral Blood and Bone Marrow Smears

Peripheral blood and bone marrow smears were made from all mice that were alive at the end of the 18-month study period. Just before necropsy, a small drop of peripheral blood was collected from each mouse (by snipping the end of the tail) and immediately placed on a clean microscope slide. The blood was pulled behind a cover slip held at a 45° angle to form a thin smear over an area of 2–3 cm. At necropsy, marrow from one femur from each mouse was flushed with 0.5 ml of fetal calf serum into a microfuge tube using a 1-ml syringe fitted with a 22-gauge needle. The cells were concentrated by gentle centrifugation at 600*g* for 2–3 min. A small drop of resuspended cells was then placed on a clean microscope slide and a smear was made as described above (*33*).

All smears were air-dried and fixed in absolute methanol. They were stained using acridine orange (0.01 mg/ml of 0.2 *M* phosphate buffer, pH 7.4), air-dried and stored in black boxes. All slides were coded by an individual other than the scorer and examined under 1000× magnification using a fluorescence microscope. Erythrocytes which were young (PCEs) were identified by their orange-red color, mature erythrocytes by their green color and the micronuclei by their yellowish color. For each mouse, the percentage of PCEs was obtained from the examination of 1.000 erythrocytes in peripheral blood and 200 erythrocytes in bone mar-

TABLE II
Micronucleus Frequencies in the Peripheral Blood and Bone Marrow of Mammary Tumor-Bearing Mice

Group	Number of mice studied	Total erythrocytes examined[a]	Group mean percentage PCEs (± SD)	Individual mouse data: micronuclei/2,000 PCEs								Group mean micronuclei/1,000 PCEs (± SD)
Peripheral blood												
Exposed to 2450 MHz RF radiation	12	12,000	2.8 (0.71)	9	10	10	9	8	10	10	7	4.6 (1.03)
				10	8	9	10					
Sham-exposed control	8	8,000	3.2 (0.44)	9	7	9	9	7	9	8	8	4.1 (0.89)
Bone marrow												
Exposed to 2450 MHz RF radiation	12	2,400	50.3 (0.73)	12	12	14	14	10	13	11	9	6.1 (1.76)
				13	13	11	15					
Sham-exposed control	8	1,600	50.2 (0.50)	12	12	11	10	9	13	9	12	5.5 (1.51)

[a]For each mouse, 1,000 consecutive erythrocytes in the peripheral blood and 200 consecutive erythrocytes in the bone marrow were examined.

row. In addition, for each mouse, in peripheral blood and in bone marrow, 2,000 consecutive PCEs were examined to determine the incidence of micronuclei. The data were analyzed using the Student's *t* test.

RESULTS

At the end of the 18-month exposure period, the study group consisted of 62 mice exposed to RF radiation, 58 sham-exposed control mice and 7 positive control sentinel mice injected with MMC. For both peripheral blood and bone marrow, the group mean percentages of PCEs among the erythrocytes and the incidence of micronuclei/1,000 PCEs are presented in Table I. The data for individual mice for the number of micronuclei/2,000 PCEs for both peripheral blood and bone marrow are also included in Table I.

The results for the group mean percentages of PCEs among the erythrocytes indicated no significant differences between RF-radiation-exposed, sham-exposed control and MMC-treated mice: The values were 2.8 ± 0.69%, 3.1 ± 0.71% and 2.7 ± 0.68% in peripheral blood, and 51.5 ± 3.80%, 52.1 ± 3.32% and 46.4 ± 3.59% in bone marrow, respectively (Table I).

The number of micronuclei/2,000 PCEs in individual mice exposed to RF radiation ranged from 7–12 in peripheral blood and 9–15 in bone marrow. A similar number of micronuclei/2,000 PCEs was observed in individual sham-exposed control mice: the range was 6–12 in peripheral blood and 8–14 in bone marrow (Table I). Thus the data did not indicate significant differences between individual mice exposed to RF radiation and sham-exposed control mice. In contrast, within 24 h of treatment with MMC, each positive control mouse had a significantly increased number of micronuclei/2,000 PCEs in peripheral blood (range 95–113) and in bone marrow (range 105–116) ($P < 0.001$).

The group mean incidence of micronuclei/1,000 PCEs in peripheral blood and bone marrow also was not significantly different between mice exposed to RF radiation and sham-exposed control mice, while the MMC-treated mice differed significantly from both mice exposed to RF radiation and sham-exposed control mice ($P < 0.001$) (Table I).

During the course of 18 months, mammary tumors were recorded in 12 of the 62 mice exposed to RF radiation and 8 of the 58 sham-exposed control mice (unpublished data). At necropsy, when the peripheral blood and bone marrow erythrocytes of only these tumor-bearing mice in each group were examined, there were again no significant differences between mice exposed to RF radiation and sham-exposed control mice; the percentages of PCEs among erythrocytes, and the incidence of micronuclei/1,000 PCEs in peripheral blood and in bone marrow, were similar in both groups (Table II).

DISCUSSION

To the best of our knowledge, this is the first reported study of the possible cytogenetic effect of chronic (lifetime) low-level 2450 MHz RF-radiation exposure in tumor-prone mice. The C3H/HeJ mice used in this study possess a milk-borne virus that induces a high incidence of tumors of the mammary gland. Because of their predisposition to develop mammary tumors, it was hypothesized that these mice might be hypersensitive to the induction of genetic damage; if the exposure to 2450 MHz RF radiation is genotoxic, the magnitude of the difference between the mice exposed to RF radiation and sham-exposed control mice could be exaggerated. In humans, it has been reported that the cells from individuals who are predisposed to the development of several malignancies are hypersensitive to the induction of damage after exposure to genotoxic mutagens (see review in ref. *34*). The results of the present study have demonstrated that the extent of genotoxicity, as measured by the incidence of micronuclei/2000 PCEs in peripheral blood and bone marrow, was not significantly different between the mice that were exposed chronically to the 2450 MHz RF radiation and the sham-exposed controls. Also, there were no significant differences between the mice exposed to RF radiation and

sham-exposed control mice regarding the percentages of PCEs in their peripheral blood and bone marrow; these data demonstrate that the division and maturation of nucleated erythropoietic cells have not been inhibited by chronic exposure to the RF radiation.

Since the mouse has often been the choice for the micronucleus assay in genotoxicity investigations, several different inbred, outbred and hybrid mouse stocks have been used in different laboratories. Recently, Salamone and Mavournin (*35*) reviewed the spontaneous frequencies of micronucleated PCEs in bone marrow of more than 55 different mouse stocks used as untreated, sham-exposed or solvent-treated controls in different investigations. Overall, the mean incidence was reported to range from 0.50–9.82 micronucleated PCEs/1,000 PCEs. At least 6 of these mouse stocks had a mean of >5 micronucleated PCEs/1,000 PCEs. The age of the mice used in majority of the studies cited in the review was less than 20 weeks. In our study, the incidence of micronucleated PCEs/1,000 PCEs in bone marrow and peripheral blood of mice exposed to RF radiation and sham-exposed control mice ranged from 4–6 micronucleated PCEs/1,000 PCEs, which is similar to the values reported by Salamone and Mavournin (*35*).

The important observation made in this investigation was that chronic low-level exposure of C3H/HeJ mice, which are prone to mammary tumors, to 2450 MHz RF radiation, the frequency which approximates maximum energy absorption for mice, did not demonstrate any genotoxicity.

ACKNOWLEDGMENTS

This work was supported by the U.S. Air Force grant No. F33615-90-0606, and partial support from the U.S. Air Force Office of Scientific Research grant No. F49620-95-1-0337. The authors gratefully acknowledge the RF-radiation exposure support of the Sources and Measurements Branch, Radiofrequency Radiation Division, USAF Armstrong Laboratory, Brooks AFB, San Antonio, TX. The animals involved in this study were procured, maintained and used in accordance with the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council. The views and opinions expressed herein are those of the authors and do not necessarily state or reflect those of the U.S. Government.

Received: July 18, 1996; accepted: October 30, 1996

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FREQUENCY OF MICRONUCLEI IN THE BLOOD AND BONE MARROW CELLS OF MICE EXPOSED TO ULTRAWIDEBAND ELECTROMAGNETIC RADIATION

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RUNNING TITLE:

Micronuclei in mice exposed to ultrawideband electromagnetic radiation.

INDEXING WORDS:

Ultrawideband Electromagnetic Radiation; Mice; Micronuclei; Peripheral Blood; Bone Marrow.

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ABSTRACT.

Purpose: To investigate the extent of genetic damage in the peripheral blood and bone marrow cells of mice exposed to ultrawideband electromagnetic radiation (UWBR).

Materials and Methods: CF-1 male mice were exposed to UWBR for 15 minutes at an estimated whole-body average specific absorption rate of 37 mW/kg. Groups of untreated control and positive control mice injected with mitomycin C were also included in the study. After various treatments, half of the mice were sacrificed at 18 hours, and the other half at 24 hours. Peripheral blood and bone marrow smears were examined to determine the extent of genotoxicity, as assessed by the presence of micronuclei (MN) in polychromatic erythrocytes (PCEs).

Results: The percentages of PCEs and the incidence of MN/2000 PCEs in both tissues in mice sacrificed at 18 hours were similar to the frequencies observed in mice terminated at 24 hours. There were no significant differences in the percent PCEs between control and the mice±UWBR exposure; the group mean values (± standard deviation) were in the range of 3.1±0.14 to 3.2±0.23 in peripheral blood, and 49.0±3.56 to 52.3±4.02 in bone marrow. The mean incidence of MN/2000 PCEs in control and mice±UWBR exposure ranged from 7.7±2.00 to 9.7±2.54 in peripheral blood, and 7.4±2.32 to 10.0±3.27 in bone marrow. Pairwise comparison of the data did not reveal statistically significant differences between controls and mice±UWBR exposed groups (excluding positive controls).

Conclusion: Under the experimental conditions tested, there was no evidence for excess genotoxicity in both peripheral blood and bone marrow cells of mice exposed to UWBR.

1. INTRODUCTION

Electromagnetic devices which are capable of producing signals with pulse widths of a few nanoseconds and electric field amplitudes exceeding 100,000 V/m are being considered in the United States and overseas for use in warfare settings, such as electronic countermeasures to identify and track incoming armor-piercing rounds that threaten ground vehicles (Taylor, 1991; Fuerer, 1991; Toevs et al., 1992). Ultrawideband electromagnetic radiation (UWBR) in the radiofrequency range is in this category of signal, and can be characterized as having a large band spread of up to 2 GHz, a rapid rise time of less than 0.2 nsec, and a pulse duration of a few nsec. It is conceivable that personnel could be exposed to such signals while operating and/or working in the vicinity of radar or communications-jamming equipment, and/or by similar friendly or enemy equipment (Sherry et al., 1995). Such potential exposure to UWBR raises issues related to possible bioeffects and/or possible human health hazard.

Recently, Albanese et al. (1994) described possible useful applications of ultra-short electromagnetic pulses in medical settings. These included electroporation, which would allow chemotherapeutic drugs to more readily enter and kill cancer cells, and development of new techniques for imaging tissue structures. The authors also associated their theoretical considerations with undesirable health effects resulting from potential tissue damage mechanisms, including macromolecular conformation changes, alterations in chemical reaction rates, membrane effects, and temperature-mediated adverse responses. Merritt et al. (1995) subsequently challenged most of these associations, and indicated the limited availability of experimental (biological) data. The very few published scientific reports have not indicated any discernible physiological or behavioral change in rats and monkeys exposed to UWBR (Walters et al., 1995; Sherry et al., 1995).

The potential of UWBR exposure to induce genetic damage, if any, is important for risk assessment related to mutation and cancer induction. Recently, Pakhomova et al. (1997, 1998) reported an absence of mutagenic influence of UWBR exposure on the D7 strain of the yeast *Saccharomyces cerevisiae.* To the best of our knowledge, an examination of genetic effects of UWBR exposure of mammalian cells, either *in vitro* or *in vivo*, has not been reported in the literature. The rodent micronucleus (MN) test has been widely accepted as an *in vivo* test system for detecting genotoxic agents, and has become a standard genetox assay used in regulatory testing in several countries (Sofuni, 1993; Kirkland, 1993; Auletta et al., 1993; Health Protection Branch Genotoxicity Committee, Canada, 1993). The objective of the present investigation is to assess the extent of genetic damage, as determined from the incidence of MN, in peripheral blood and bone marrow cells of mice exposed to UWBR for 15 minutes.

2. MATERIALS AND METHODS

A protocol approved by the Institutional Animal Care and Use Committee of the United States Air Force Armstrong Laboratory, Brooks Air Force Base, was followed. The Standard Operating Procedures were compatible with the requirements of the United States National Toxicology Program.

2.1. UWBR Exposure

The UWBR exposure facility is located at Brooks Air Force Base in San Antonio. Mice were exposed to UWBR pulses in a giga-transverse-electromagnetic (GTEM) cell comprised of a tapered rectangular coaxial transmission line with a square cross section (originally constructed by Sandia National Laboratory, Albuquerque, New Mexico). The outer ground conductor had a square cross-section measuring 11-71 cm inside the cell (the range results from the tapered structure of the cell). The center

conductor was 7.8-56.3 cm wide in the same region, and was 2.6 cm thick throughout the cell. An approximate volume of 20x20x40 cm was available on each broad side of the center conductor for placing a single mouse in a circular plastic holder. A modified RG-220 coaxial cable connected the GTEM cell to a source of high voltage. Ionization of pressurized nitrogen gas in a spark gap in the cable resulted in high voltage pulses which led to UWBR pulses in the GTEM cell. In this system, the electric field was directed from the center conductor to the ground conductor.

The propagating UWBR pulses in the GTEM cell were monitored during exposures of mice using signals from a time-derivative (D-dot) probe mounted in the wall of the cell. Stored wave forms were later processed using a correction algorithm to give the electric field strength versus time (Bao, 1997). Pulses were triggered at 600 pulses/sec by an external pulse generator. The peak amplitude of the UWBR pulse was 91.1-102.9 kV/m at the location of the center of the animal holder. The pulse rise time was 146.6-166.1 psec and the pulse duration was 0.92-0.97 nsec. The whole body specific absorption rate (SAR) was estimated to be 37 mW/kg. This was done by integrating the product of the power spectrum, computed from the corrected UWBR pulse field strength, and the normalized SAR (W/kg per mW/cm^2) for a prolate spheroidal model of a medium-sized mouse (Durney et al., 1986) in the frequency domain. The average for k- and H-polarizations of the animal was used to account for the movement of the animal in the UWBR field.

2.2. Mice and Maintenance

Ten- to twelve week-old CF-1 male mice were obtained from Charles River Laboratories, Portage, Michigan; the mice weighed 33-48 grams. Upon arrival, they were housed 2-4 per cage in an animal facility at Brooks Air Force Base. The room was maintained at a temperature of $22\pm1^{\circ}C$ and a relative humidity of $50\pm5\%$, with an air-flow rate of 10-15 exchanges/hour. A time-controlled system provided a daily 0500-1700 hour light and 1700-0500 hour

dark cycle. All mice were given *ad libitum* access to Purina rodent chow and to tap water.

After a 10-day quarantine period, a total of 61 mice were distributed into separate groups using a randomized block design. There were 9 mice in the untreated control group, 12 mice in the positive control group, and 10 mice in each of ±UWBR exposure groups. Each mouse was placed in a circular plastic holder that did not restrict the movement of the animal. The mouse in its holder was placed in the GTEM cell for UWBR exposure for 15 minutes at 600 pulses/sec (+UWBR), or to no pulses (-UWBR); the exposure was done at room temperature, one mouse at a time. The selection of the 15 minute duration of the UWBR exposure was based on an earlier observation (unpublished) which indicated a potential UWBR exposure time-related effect on morphine-induced analgesia in these mice. After each UWBR exposure, the holder was removed from the GTEM cell and the mouse was taken. All mice were returned to their cages and kept in the animal facility until sacrifice at 18 or 24 hours when the peripheral blood and bone marrow cells were collected for the genetox study reported here.

2.3. Positive Controls

Mice in this group were given an i.p. injection of mitomycin C (MMC, 1 mg/kg body weight) (Sigma, St. Louis, MO) at 18 or 24 hours before sacrifice. MMC is a known clastogen which has been shown to induce MN (Heddle et al., 1984).

2.4. Peripheral Blood and Bone Marrow Smears

From each group, half of the mice were sacrificed at 18 hours, and the remaining half were terminated at 24 hours. From each mouse, before sacrifice, a small drop of peripheral blood was collected by snipping the end of the tail, and immediately placed on a clean microscope slide. A thin smear was made over an area of 2-3 cm by pulling the blood behind a coverglass held at a 45° angle. Also from each mouse after sacrifice, bone marrow from

both femurs was flushed with 0.5 ml of fetal calf serum into a microfuge tube using a 1 ml syringe fitted with a 22G needle. The cells were concentrated by gentle centrifugation at 600g for 1-3 minutes and a small drop of resuspended cells was placed on a clean microscope slide to make a smear as described above. All smears were air-dried, fixed in absolute methanol, and stained using acridine orange.

Coded slides were examined under 1000x magnification using a fluorescence microscope equipped with appropriate filters. Erythrocytes which were young (PCEs) were identified by their orange-red color, mature erythrocytes by their green color, and the MN by their yellowish color. For each mouse, 1000 erythrocytes in peripheral blood and 200 erythrocytes in bone marrow were examined to obtain the percentage of PCEs. In addition, for each mouse, 2000 consecutive PCEs were examined in peripheral blood and in bone marrow to determine the incidence of MN. Decoding of the slides was done after completing the microscopic analysis.

2.5. Statistical Analysis

The statistical methods used were descriptive statistics of mean, standard deviation and analysis of variance of the groups used in the experiment. Pairwise multiple comparisons of individual means of different groups were done to compare the controls, ±UWBR exposed and MMC group for sacrifice times at 18 and 24 hours. Residuals were analyzed to determine whether the best skewness and kurtosis were with the raw data or the usual transformations for small percentages and for small frequency counts (Zar, 1974). In addition, deleted residuals versus raw residuals were plotted to verify that no one observation had undue influence on the results. A final plot of residuals versus predicted values was used to verify normality of distribution, homogeneity of variance, and lack of outliers, yielding a valid analysis.

3. RESULTS

The mean percentages of PCEs and the average incidence of MN/2000 PCEs (± standard deviation) for the mice in control, ±UWBR exposed, and the positive control groups (sacrificed at 18 and 24 hours) are presented in Table 1.

3.1. Peripheral Blood

The percentages of PCEs in control and ±UWBR exposed mice sacrificed at 18 and 24 hours were all within the range of 3.1±0.14 to 3.2±0.23 (p=0.1326 for the overall effect of UWBR). The positive control mice injected with MMC exhibited decreased percentages of PCEs when sacrificed at 18 hours (2.9±0.14) and at 24 hours (2.6±0.17).

The frequencies of MN/2000 PCEs in control mice sacrificed at 18 hours and at 24 hours were 8.3±3.30 and 9.2±2.68, respectively. The indices for MN/2000 PCEs in ±UWBR exposed mice terminated at 18 and 24 hours were similar, ranging from 7.7±2.00 to 9.7±2.54 (p=0.0975 for the overall effect of UWBR). The positive control mice treated with MMC exhibited a significantly increased incidence of MN/2000 PCEs when sacrificed at both 18 hours (99.2±3.31) and at 24 hours (107.3±8.07) (p=0.0001).

3.2. Bone Marrow

The percentages of PCEs in control mice sacrificed at 18 hours and at 24 hours were 49.0±3.56 and 52.3±4.02, respectively. In ±UWBR exposed mice, the percent PCEs (at both times of sacrifice) ranged between 50.0±1.42 and 51.4±3.04 (p=0.7052 for the overall effect of UWBR). The positive control mice exhibited a decrease in the percentage of PCEs when sacrificed at 18 hours (44.6±3.07) and at 24 hours (42.3±2.14).

The frequencies of MN/2000 PCEs in control mice sacrificed at 18 hours and at 24 hours were 8.8±2.06 and 9.8±2.86, respectively.

The indices for MN/2000 PCEs in ±UWBR exposed mice terminated at 18 and 24 hours ranged between 7.4±2.32 and 10.0±3.27 (p=0.0722 for the overall effect of UWBR). The positive control mice treated with MMC exhibited significantly increased frequencies of MN/2000 PCEs when sacrificed at both 18 hours (102.7±9.09) and at 24 hours (114.8±14.88) (p=0.0001).

4. DISCUSSION

In mammals during erythroblastosis, for a still unknown reason, the main nucleus is expelled and lagging chromosomal fragments and/or whole chromosomes that are not incorporated into daughter nuclei during cell division persist as easily recognizable micronuclei in young erythrocytes (PCEs). The first appearance of MN in PCEs occurs 10-12 hours after a clastogenic exposure. This lag period results from the time required for the erythroblast to divide, to expel its main nucleus to become the polychromatic erythrocyte, and any mitotic delay induced by the genotoxic agent (Heddle et al., 1984). Once induced, the MN persists in the PCEs for about 30 hours (Jenssen and Ramel, 1980). Hence, the clastogenic and/or aneugenic effect of a given treatment can be detected at any point during this time interval (Heddle et al., 1984). The main requirement of the treatment/sampling schedule is to obtain at least one sample at or near the time of the maximum incidence of micronucleated PCEs (MacGregor et al., 1987). In the present study, peripheral blood and bone marrow cells were examined at 18 hours and at 24 hours following UWBR exposure.

There were no significant differences in the percent PCEs between control and mice exposed to ±UWBR and indicate that the time required for nucleated erythropoietic cells to become PCEs is not altered by *in vivo* exposure of mice to ±UWBR used in this study: a marked reduction in the frequency of PCEs would have

indicated that the division and maturation of the nucleated erythropoietic cells have been inhibited (MacGregor et al., 1987).

The incidence of MN observed in the bone marrow cells of control mice were comparable to those reported earlier for adult CF-1 mice (average of 4 MN/1000 PCEs) (Okine et al., 1983). The influence of UWBR exposure on the incidence of MN/2000 PCEs in both the peripheral blood and bone marrow cells determined by pairwise analysis did not indicate significant differences between control and ±UWBR exposed mice. In recently published genetox investigations, Pakhomova et al. (1997) observed no significant difference in the occurrence of chromosome recombinations, mutations, and abnormal colonies (i.e., mitotic crossovers, segregations, revertants and convertants) between UWBR-exposed and sham-exposed D7 strain of yeast *saccharomyces cerevisiae*; in that study, the UWBR exposure was for 30 minutes, with a pulse repetition rate of 16-600 Hz, a pulse duration of 1.01-1.02 nsec, a pulse rise time of 164-166 psec. This gives a bandwidth of 190% (Foster et al., 1995) and a peak electric field strength of 101-104 kV/m. In a subsequent paper, the same authors reported no significant effect of similar UWBR exposure on the incidence of ultraviolet light (UV) (2.25 J/m^2/sec and a total exposure of 100 J/m^2) induced reciprocal and non-reciprocal recombination or mutagenesis, or on UV-induced cytotoxicity (Pakhomova et al. 1998).

Earlier investigations in rats, mice and monkeys exposed to UWBR did not demonstrate any altered physiological responses. Walters et al. (1995) reported no significant differences in a swimming performance test, in blood chemistry, or in the expression of c-fos protein in brain cells, between control rats and those exposed to UWBR for 2 minutes (pulse repetition rate 60 Hz, pulse duration 7-8 nsec, band width 0.25-2.5 GHz, peak E-field strength 250 kV/m, and far field equivalent peak power density of 1.7×10^9 W/cm^2). In a recent report, Jauchem et al. (1997) indicated no significant differences in heart rate and arterial blood pressure between UWBR-exposed (pulse repetition rate 1 kHz,

pulse rise time 300 psec, and E-field strength 21 kV/m) and control rats. Sherry et al. (1995) exposed six monkeys to UWBR for 2 minutes (pulse frequency 60 Hz, pulse duration 5-10 nsec, a total of 7200 pulses, band-width 100 MHz to 1.5 GHz, peak E-field strength of 250 kV/m, and a whole-body SAR calculated to be 0.5 mW/kg). Each monkey was exposed to UWBR twice, with an interval of 6 days between exposures. The mean primate equilibrium platform performance for all monkeys after UWBR exposure was not different than that observed before each UWBR exposure. Seaman et al. (1998) reported that exposure of mice to UWBR for 30 minutes (60-600 pulses/sec, pulse duration 1.00 nsec, rise time 200 psec, pulse amplitude 100 kV/m and a calculated band width of 184.3% (Foster et al., 1995) did not result in significant changes in normal or morphine-induced nociception and locomotor activity. In contrast, an *in vitro* exposure of murine macrophages to UWBR (with similar exposure conditions), under certain conditions of macrophage stimulation, resulted in an increase in the production of nitric oxide (Seaman et al., 1996b).

The results from this investigation did not indicated excess genotoxicity in both peripheral blood and bone marrow cells of mice exposed to UWBR for 15 minutes.

ACKNOWLEDGMENTS

This work was supported by the United States Army Medical Research and Material Command under contract DAMD17-94-C-4069 awarded to McKesson BioServices, and by the United States Air Force Office of Scientific Research grant No. F49620-95-1-0337. We thank T. -S. Lu for discussions on the estimate of SAR and and W. D. Hurt for SAR calculation. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation. In conducting the research using animals, the investigator(s) have adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publ. # 86-23, Rev. 1985).

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Table 1. The percentages of polychromatic erythrocytes (PCEs) and the incidence of micronuclei (MN) in the peripheral blood and bone marrow cells of mice exposed to ultrawideband electromagnetic radiation (UWBR) for 15 minutes.

Group	Group Mean % PCEs (+/- Std. Dev.)	Group Mean MN/2000 PCEs (+/- Std. Dev.)
Peripheral Blood		
Mice sacrificed at 18 hours after UWBR exposure		
Controls	3.1 (0.17)	8.3 (3.30)
UWBR-	3.2 (0.23)	7.7 (2.00)
UWBR+	3.1 (0.17)	9.7 (2.54)
Mitomycin C	2.9 (0.14)	99.2 (3.31)
Mice sacrificed at 24 hours after UWBR exposure		
Controls	3.1 (0.14)	9.2 (2.68)
UWBR-	3.2 (0.23)	8.8 (2.20)
UWBR+	3.1 (0.20)	9.4 (2.88)
Mitomycin C	2.6 (0.17)	107.3 (8.07)
Bone marrow		
Mice sacrificed at 18 hours after UWBR exposure		
Controls	49.0 (3.56)	8.8 (2.06)
UWBR-	51.4 (3.04)	7.4 (2.32)
UWBR+	51.2 (2.61)	8.7 (2.00)
Mitomycin C	44.6 (3.07)	102.7 (9.09)
Mice sacrificed at 24 hours after UWBR exposure		
Controls	52.3 (4.02)	9.8 (2.86)
UWBR-	50.5 (3.55)	8.1 (2.28)
UWBR+	50.0 (1.42)	10.0 (3.27)
Mitomycin C	42.3 (2.14)	114.8 (14.88)

* For each mouse, 1000 consecutive erythrocytes in the peperipheral blood and 200 consecutive erythrocytes in the bone marrow were examined.

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June 7-11, 1998

plexes) in the liver and tumor tissues were estimated by using EPR spectroscopy. All experiments were done under blind conditions. The obtained data were statistically evaluated by using non-paramagnetic methods.
SUMMARY: The treatment of animals with the modified endogenous EMFs resulted in a statistically significant decrease in the rate of tumor development. The analysis of air dried preparations of tumor tissue in mice showed that the proliferation of tumor cells decreased after BRT. The number of animals with metastases as well as the mean value of the number of metastases per animal decreased in the treated groups. Tumor development results in a decrease in the content of free radicals in the tissues of tumor-bearing mice as compared with that of intact animals, whereas BRT of experimental animals resulted in an increase in free radical concentration in the liver of mice. The increased level of semiquinone forms of flavines and flavoproteins in the liver mat testify to the BRT-induced adaptation to the changes in the oxidative pathways in mitochondria that were generated by tumor development. The changes in the intensity of the oxidized iron signal testify to that the observed tumor cell cytotoxicity of BRT may be well due to iron release under the appropriate EMF influence.
CONCLUSIONS: The standard development of the malignant process is changed under the influence of the endogenous electromagnetic fields of tumor and adjacent-to-tumor tissues: the development of melanoma B16 in mice is inhibited as well as the metastasizing spreading in the lung of the experimental animals is decreased. This may reflect the restoration of the physiological informational pathways in the organism under the influence of appropriate EMF treatment. The application of the endogenous electromagnetic fields ensures the conditions of biological resonance and thus facilitates the search for the appropriate regime of EMF treatment.

P-60B

FREQUENCY OF MICRONUCLEI IN THE PERIPHERAL BLOOD OF MICE EXPOSED TO ULTRA WIDEBAND MICROWAVE RADIATION. Vijayalaxmi*[1], R.L. Seaman[2] and M.L. Meltz[1].
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CF-1 male mice were exposed to ultrawideband electromagnetic radiation (UWBR) for 15 minutes at an estimated whole-body average specific absorption rate of 11 mW/kg. Experimental mice were injected with either morphine (7.5 mg/kg body weight) or saline one minute before UWBR (±) exposure. Groups of untreated control and positive control mice (injected with mitomycin C) were also included in the study. After various treatments, half of the mice were sacrificed at 18 hours, and the other half at 24 hours. Peripheral blood smears were examined to determine the extent of genotoxicity, as assessed by the presence of micronuclei (MN) in polychromatic erythrocytes (PCE). The percentages of PCE and the incidence of MN/2000 PCE in mice sacrificed at 18 hours were similar to the frequencies observed in mice terminated at 24 hours. There were no significant differences in the percent PCE between untreated controls and the experimental mice which were injected with morphine ± UWBR and saline ± UWBR; the group mean percent values (± S.D.) were in the range of 3.0±0.23 to 3.2±0.23. The mean incidence of MN/2000 PCE in untreated and experimental mice ranged from 7.7±2.00 to 11.6±3.34. Pairwise comparison of the data did not reveal statistically significant differences between any UWBR exposed group and any other individual groups (excluding positive controls). For consolidated data, (i) the influence of treatment with morphine (all mice injected with morphine) versus saline (all mice treated with saline) did not indicate significant differences between the two groups, and (ii) the influence of UWBR exposure (all mice exposed to UWBR versus all mice which were not exposed to UWBR) did indicate a statistically significant increase of 1.27 MN/2000 PCE in exposed group over unexposed group: this needs confirmation in further investigations. The small size of the change, observed only from the consolidated data of UWBR exposed group, requires that the biological relevance be put in proper perspective.

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Mutation Research 397 (1998) 203–208

Fundamental and Molecular Mechanisms of Mutagenesis

Melatonin reduces gamma radiation-induced primary DNA damage in human blood lymphocytes

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Received 27 June 1997; revised 20 August 1997; accepted 27 August 1997

Abstract

Peripheral blood samples were collected from human volunteers 5–10 min before, and at 1 and 2 h after a single oral dose of 300 mg of melatonin. At each time point: (1) the concentration of melatonin in the serum and in the leukocytes was determined; and (2) the whole blood was exposed in vitro to 100 cGy of gamma radiation. Immediately after exposure to the radiation, the lymphocytes were examined to determine the extent of primary DNA damage, viz., single strand breaks and alkali labile lesions (determined from the length of DNA migration and fluorescence intensity of migrated DNA in the comet tail), using the alkaline comet assay. For each volunteer, the results showed a significant increase in the concentration of melatonin in the serum and in the leukocytes at 1 h after the oral dose of melatonin, as compared to the sample collected at 0 hour. The lymphocytes in the blood samples collected at 1 and 2 h after melatonin ingestion and exposed in vitro to 100 cGy gamma radiation exhibited a significant decrease in the extent of primary DNA damage, as compared with similarly irradiated lymphocytes from the blood sample collected before melatonin ingestion. The extent of the melatonin-associated decrease in primary DNA damage did not correspond with the decrease reported earlier in the incidence of chromosomal aberrations and micronuclei: the latter assays required an additional postirradiation incubation of the cells at 37 ± 1°C for 48 and 72 h, respectively. © 1998 Elsevier Science B.V.

Keywords: Melatonin; Gamma radiation; Primary DNA damage; Comet assay; Radioprotection

1. Introduction

Melatonin, a secretory product of the pineal gland in human brain, has been reported to participate in the regulation of a number of important physiological and pathological processes [1–3]. Recently, we have reported a significant reduction in the incidence of chromosomal aberrations and micronuclei in human blood lymphocytes which were pretreated in vitro with melatonin and then exposed in vitro to 150 cGy gamma radiation, as compared with similarly irradiated cells which were not pretreated with melatonin [4,5]. A subsequent study involved an in vivo melatonin ingestion followed by an in vitro radiation exposure of blood samples from 4 different

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PII S0027-5107(97)00211-X

human volunteers. The results from this study indicated that following the ingestion of a single oral dose of 300 mg of melatonin, the concentration of melatonin in the serum and in leukocytes was increased significantly in all volunteers. The lymphocytes which were exposed in vitro to 150 cGy gamma radiation at 1 and 2 h after the oral ingestion of melatonin (and then mitogenically stimulated, and cultured for 48 or 72 h) exhibited a significantly decreased incidence of chromosomal aberrations and micronuclei, as compared with similarly irradiated cells sampled before the ingestion of melatonin [6].

These results could be due to a significant reduction by melatonin of the extent of the primary DNA damage induced by the indirect action of gamma radiation. Alternatively, melatonin might have activated cellular DNA repair enzymes, facilitating a more rapid repair of the damaged DNA [4,6]. Neither of the two genetic end-points studied earlier, by themselves, provided information that could assist in the examination of these two hypotheses, since the observations of microscopically visible chromosomal aberrations and micronuclei were made 48 and 72 h after irradiation, respectively.

To examine the first of these proposed hypotheses, small aliquots of each of the peripheral blood samples collected during the earlier in vivo/in vitro study in human volunteers were utilized [6]. The alkaline comet assay [7–11] was used to determine the extent of DNA damage, viz., single strand breaks and alkali labile lesions, in the lymphocytes collected from human volunteers at 0 h (before melatonin ingestion), and at 1 and 2 h following the ingestion of a single oral dose of 300 mg of melatonin, and immediately after irradiation at those times.

2. Materials and methods

A protocol approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio was followed to obtain the peripheral blood samples from 4 healthy, non-smoking human volunteers, two males and two females aged between 30 and 53 years. After overnight fasting, each volunteer was given a single oral dose of a gelatin capsule containing 300 mg of melatonin (Regis Chemical Co., Morton Grove, IL, 99.9% purity) at 08.45 h. Peripheral blood samples were collected in sterile vacutainer tubes ± heparin as anticoagulant, at 0 (5–10 min before the ingestion the melatonin), and at 1 and 2 h (±5 min) after the oral dose of melatonin.

For each volunteer, at each of the collection times, serum was separated from the blood in the vacutainer tubes without heparin, and 5 ml of heparinized blood was used to separate the leukocytes over a ficoll-hypaque gradient. The concentration of melatonin in all serum and leukocytes samples was determined using a highly specific antibody (Guildhay Stockgrand Antisera, Guilford, UK) in a direct radioimmunoassay [12] as described previously [6].

At each of the collection times, for each volunteer, duplicate aliquots of 500 μl of heparinized whole blood were placed in 1.5 ml microfuge tubes kept on ice (to prevent the repair of DNA damage) and exposed in vitro to 100 cGy gamma radiation from a ^{137}Cs source (GammaCell-40 Irradiator, Atomic Energy of Canada Ltd.; dose rate of 108.7 cGy/min). Similar aliquots of blood samples were used as unirradiated controls. Immediately after irradiation, from each aliquot, approximately 7 μl of whole blood was mixed with 75 μl of 0.5% low melting point agarose (kept at 37 ± 1°C) and microscope slides were prepared for the comet assay; during this step of the process, the cells were at 37 ± 1°C for less than 20 s. The technique developed by Singh et al. [7] was followed with minor modifications, as described in detail earlier [8]. All slides were immersed in a jar containing cold lysing solution and left at 4 ± 1°C for at least 1 h. The slides were then treated with DNAse free proteinase K (Boehringer Mannheim, Indianapolis, IN, 1 mg/ml) for 1 h at 37 ± 1°C [13], placed on the platform of a horizontal electrophoresis box, and exposed to an alkaline buffer (pH 13.0) for 20 min. An electric current of 25 V and 300 mA was then applied for 20 min to electrophorese the DNA. The slides were then treated gently with 0.4 M Tris buffer (pH 7.5) to neutralize excess alkali, and stained with 50 μl of a 2 μg/ml ethidium bromide solution (Sigma, St. Louis, MO).

Coded slides were examined at 250× magnification using a Zeiss fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. From each blood sample, images of

50 randomly selected nuclei (25 nuclei from each of the duplicate aliquots) were analyzed using a semi-automated Komet Image Analysis System (Kinetic Imaging Ltd., Liverpool, UK, Version 3) linked to a CCD camera. For each nucleus, the total length of the image (including nucleus and tail) and the diameter of the nucleus were measured in microns; the fluorescence intensity of the migrated DNA in the comet tail (arbitrary units) was also recorded. The extent of DNA damage was assessed from: (1) the length of DNA migration (derived by subtracting the diameter of the nucleus from the total length of the image); and (2) the fluorescence intensity in the comet tail.

For each volunteer, at each blood collection time, the extent of radiation-induced DNA damage was computed by subtracting the values obtained in corresponding unirradiated control lymphocytes from those recorded for irradiated lymphocytes. The difference in the extent of radiation-induced DNA damage between 0 h, and 1 and 2 h after melatonin ingestion, was verified by Student's *t*-test.

3. Results

None of the volunteers indicated any obvious adverse side effects from the ingestion of a single oral dose of 300 mg melatonin. As previously reported, for each volunteer, the concentrations of melatonin in the serum and in the leukocytes of the blood samples collected at 1 and 2 h after melatonin ingestion were significantly higher than in those sampled at 0 h (Table 1) (data taken from [6]).

The responses of the lymphocytes from all 4 volunteers to in vitro gamma radiation exposure at 0 h (before melatonin ingestion), and at 1 and 2 h after the oral dose of melatonin were similar, although the absolute values (mean ± SE) were slightly different. In all blood samples, for all unirradiated control cells, the lengths of DNA migration ranged between 21.3 ± 0.36 and 26.5 ± 0.51 μm. In contrast, all lymphocytes exposed in vitro to 100 cGy gamma radiation exhibited significantly increased lengths of DNA migration; the values ranged between 68.3 ± 1.22 and 104.9 ± 2.04 μm. The data presented in

Table 1
Gamma radiation-induced primary DNA damage in blood lymphocytes from human volunteers examined at 0 h (5–10 min before), and at 1 and 2 h (±5 min) after a single oral ingestion of 300 mg of melatonin[a]

Blood collection time + radiation dose	Melatonin concentration		Length of DNA migration (μm)		Fluorescence intensity in Comet tail (arbitrary units)	
	Serum (ng/ml)	Leukocytes (ng/mg protein)	Mean ± SE	% Decrease	Mean ± SE	% Decrease
Volunteer 1						
0 h + 100 cGy	0.04	0.01	71.8 ± 2.40		24.1 ± 0.99	
1 h + 100 cGy	0.96	0.44	60.6 ± 1.70	15.6[b]	21.0 ± 0.69	12.9[b]
2 h + 100 cGy	83.13	0.07	55.6 ± 1.60	22.6[b,c]	18.5 ± 0.65	23.2[b,c]
Volunteer 2						
0 h + 100 cGy	0.04	0.01	67.4 ± 2.21		22.6 ± 0.96	
1 h + 100 cGy	1.37	29.69	54.2 ± 1.25	19.6[b]	19.5 ± 0.76	13.7[b]
2 h + 100 cGy	0.86	33.55	50.0 ± 1.51	25.8[b,c]	17.2 ± 0.78	23.9[b,c]
Volunteer 3						
0 h + 100 cGy	0.04	0.01	59.2 ± 2.24		20.0 ± 1.00	
1 h + 100 cGy	80.95	30.45	46.8 ± 1.35	20.9[b]	15.9 ± 0.87	20.5[b]
2 h + 100 cGy	1.70	1.27	46.2 ± 1.41	22.0[b]	15.2 ± 0.78	24.0[b]
Volunteer 4						
0 h + 100 cGy	0.04	ND	83.2 ± 1.99		28.7 ± 1.02	
1 h + 100 cGy	85.41	ND	69.2 ± 1.42	16.8[b]	24.2 ± 0.54	15.7[b]
2 h + 100 cGy	1.38	ND	67.3 ± 1.17	19.1[b]	23.4 ± 0.47	18.5[b]

[a] The values for the corresponding unirradiated controls have already been subtracted.
[b] Significant difference between 0, and 1 or 2 h. $p < 0.01$.
[c] Significant difference between 1 and 2 h. $p < 0.025$.

Table 1 indicates that the irradiated lymphocytes from all 4 volunteers which were examined at 1 and 2 h after the oral ingestion of melatonin exhibited decreased lengths of DNA migration as compared with those irradiated cells collected at 0 h; the percent decreases were between 15.6 and 20.9% at 1 h, and between 19.1 and 25.8% at 2 h ($p < 0.01$). For volunteers 1 and 2, the irradiated cells examined at 2 h after the oral dose of melatonin showed an additional decrease in the length of DNA migration, as compared with those sampled at 1 h ($p < 0.025$); the same was not the case for volunteers 3 and 4.

The fluorescence intensity measured in the comet tail (mean ± SE) for all unirradiated control lymphocytes ranged between 10.6 ± 0.24 and 12.8 ± 0.25 (Table 1). The values were significantly increased for all lymphocytes exposed in vitro to 100 cGy gamma radiation, and ranged between 26.5 ± 0.83 and 39.4 ± 0.97. The data from both unirradiated control and irradiated lymphocytes of all volunteers, at each of the blood collection times, indicated a positive correlation of fluorescence intensity with the length of DNA migration previously described. The data given in Table 1 indicates that the irradiated cells examined at 1 and 2 h after the ingestion of melatonin exhibited decreased fluorescence intensity in the comet tail as compared with the irradiated cells collected at 0 h; the percent decreases were

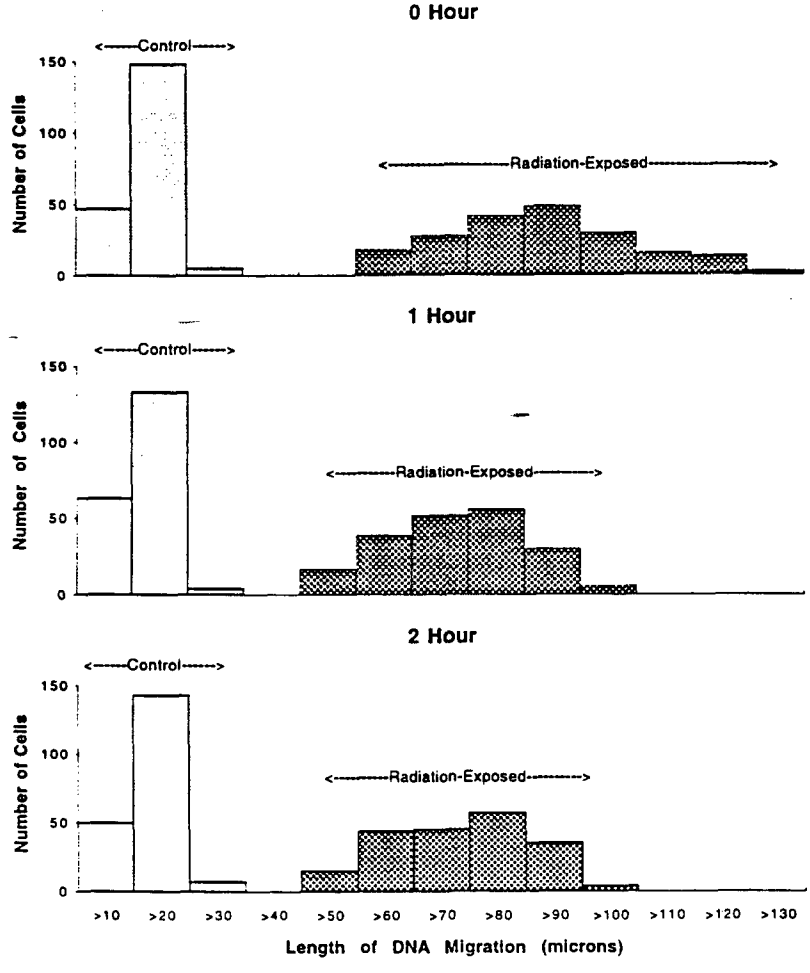

Fig. 1. Numbers of cells with different lengths of DNA migration in the blood samples collected from 4 human volunteers at 0 h (5–10 min before), and at 1 and 2 h (±5 min) after a single oral dose of 300 mg of melatonin.

between 12.9 and 20.5% at 1 h, and between 18.5 and 24.0% at 2 h ($p < 0.01$). As in the case for the length of DNA migration, for volunteers 1 and 2, the irradiated cells examined at 2 h after the oral dose of melatonin showed an additional decrease in fluorescence intensity, as compared with those sampled at 1 h ($p < 0.025$).

The data indicating the numbers of control and irradiated lymphocytes exhibiting various lengths of DNA migration were pooled from all 4 volunteers for each of the blood collection times. These data are graphically represented in Fig. 1. The distribution of the control cells with respect to different lengths of DNA migration were similar at all blood collection times. In contrast, the numbers of irradiated cells with increased lengths of DNA migration were obviously shifted to shorter lengths at 1 and 2 h after melatonin ingestion.

4. Discussion

The increased concentration of melatonin in the serum and leukocytes of the blood samples collected at 1 and 2 h following the ingestion of melatonin indicated a considerable individual- and time-related variation among the 4 volunteers. This may be due to differences between individual rates of absorption and/or clearance of melatonin from blood circulation.

In normal human blood lymphocytes, the extent of primary DNA damage induced by gamma radiation was reported to increase linearly with increasing dose [8,14], and the extent of induction was shown to be variable among the (three) individuals investigated [14]. Similar variability in the extent of 100 cGy gamma radiation-induced primary DNA damage was observed in the 4 volunteers examined in this study.

The increased concentration of melatonin in the serum and/or leukocytes showed a positive correlation with: (1) decreased extent of primary DNA damage (length of DNA migration and the fluorescence intensity); and (2) increased numbers of cells with decreased extent of DNA damage. It is also interesting to note that the two volunteers who had the highest concentrations of melatonin in the serum (volunteer 1) and leukocytes (volunteer 2) of the blood samples collected at 2 h after the oral dose of melatonin showed an additional reduction in the extent of DNA damage as compared with their cells sampled at 1 h ($p < 0.025$).

The small size and high lipophilicity of the melatonin molecule readily permits its diffusion through biomembranes [15]. In mammalian tissues, melatonin (exogenously administered or endogenously synthesized) has been shown to concentrate more in the nucleus than in the cytosol of the cell [16]. It has been reported that melatonin has the ability to scavenge hydroxyl radicals [17], peroxyl radicals [18] and peroxynitrite anions [19], and to act as an antioxidant [20]. Melatonin appears to inactivate the hydroxyl radicals by electron donation; it becomes an indolyl (melatonyl) cation radical which catalytically scavenges the superoxide anion radical and produces the product N^1-acetyl-N^2-formyl-5-mehoxy kyaramine, which is excreted in the urine [21]. In addition, melatonin is reported to have membrane and nuclear receptor-mediated, as well as receptor-independent actions [22–24]. All these properties of melatonin might have played a role in reducing the extent of gamma radiation-induced primary DNA damage in the blood lymphocytes.

In this study, the 100 cGy radiation dose was used because of the constraint of the increased sensitivity of the comet assay when evaluating primary DNA damage. It is unclear how the 13–25% decrease in the primary DNA damage after 100 cGy observed in this study as a result of melatonin ingestion can be related to the 50–60% decrease in the incidence of chromosomal aberrations and micronuclei previously recorded after 150 cGy exposure of the same population of blood lymphocytes [6]. In the latter studies, the lymphocytes were cultured at 37 ± 1°C for 48 and 72 h to record the incidence of chromosomal aberrations and micronuclei, respectively [6]. It appears that melatonin is altering some other biochemical process(es), and that these processes are also exerting an influence (during the post-irradiation culture period) on the decreased radiation-induced chromosomal damage. Possibly, melatonin can facilitate a more efficient repair of those primary strand breaks in the DNA which lead to chromosomal damage. The mechanism(s) of this interesting difference between primary DNA damage and chromosomal damage remains to be determined.

Acknowledgements

This research was supported by the Division of Radiation Oncology, The University of Texas Health Science Center, and grants from United States Air Force Office of Scientific Research (F49620-95-1-0337) and National Institutes of Health (ES07132).

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MELATONIN AND PROTECTION FROM WHOLE-BODY IRRADIATION: SURVIVAL STUDIES IN MICE

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Number of Copies Submitted	:	5
Number of Tables	:	1
Number of Figures	:	1

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RUNNING TITLE:

Melatonin and survival of whole-body irradiated mice.

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KEY WORDS:

Melatonin
Radioprotection
Gamma Radiation
Survival

ABSTRACT

Vijayalaxmi, Martin L. Meltz, Russel J. Reiter, Terence S. Herman and Sree Kumar K. Melatonin and protection from whole-body irradiation: Survival studies in mice. *Radiat. Res.*

The radioprotective ability of melatonin was investigated in mice exposed to an acute whole-body gamma radiation dose of 815 cGy (estimated $LD_{50/30}$ dose). The animals were observed for mortality over a period of 30 days following irradiation. The results indicated 100% survival for unirradiated and untreated control mice, and for mice treated with melatonin or solvent alone. Forty-five percent of mice exposed to 815 cGy radiation alone, and 50% of mice pre-treated with solvent and irradiated with 815 cGy were alive at the end of 30 days. In contrast, 60% and 85% of irradiated mice which were pre-treated with 125 mg/kg ($p=0.3421$) and 250 mg/kg melatonin ($p=0.0080$), respectively, were alive at the end of 30 days. These results indicate that melatonin (at a dose as high as 250 mg/kg) is non-toxic, and that high doses of melatonin are effective in protecting mice from lethal effects of acute whole-body irradiation.

INTRODUCTION

The ability of certain substances to provide protection against the damaging effects of ionizing radiation was first noted in 1942 and published in 1949 (1). The most remarkable radioprotectors are the sulfhydryl compounds, such as cysteine (2) and cysteamine (3). However, these compounds appear to produce serious side effects and are considered to be toxic at the doses required for radioprotection (4). The Walter Reed Army Research Institute in the United States synthesized many compounds in an attempt to find a useful radioprotector, one that would protect against ionizing radiation without toxic side effects. The most effective compound of this type, originally tested against lethal doses of X- and gamma rays in mice, was WR-2721 (which is known as amifostine). Although WR-2721 was reported to be tolerated well in clinical trials, it still had a number of undesirable side effects. These include nausea, vomiting, sneezing, hot flashes, mild somnolence, hypocalcemia and hypotension. These side effects are severe enough to limit the amount of the drug tolerated to levels lower than necessary to achieve maximum radioprotection (5-8). Moreover, WR-2721 must be given intravenously, which limits its utility outside of controlled clinical situations. Thus, there is still a need to identify non-toxic, effective and convenient compounds to protect humans against radiation damage.

Melatonin, an endogenous compound synthesized by the pineal gland in the human brain, has been reported to participate in the regulation of a number of physiological and pathological processes (9-13). Melatonin has also been shown to scavenge hydroxyl (14-16) and peroxyl radicals (17), and peroxynitrite anion (18), and to act as an antioxidant (19). In *in vitro* investigations, human peripheral blood lymphocytes which were pre-treated with melatonin were found to a exhibit significantly reduced (60-70%) incidence of gamma radiation-induced chromosomal damage as compared with those irradiated cells which were not pre-treated with melatonin (20, 21). Lymphocytes from human volunteers who ingested a single

oral dose of 300 mg of melatonin (one hour prior to collection of their blood) were also shown to exhibit a significantly reduced (60-65%) extent of genetic damage after the subsequent exposure of the cells *in vitro* to gamma radiation (22). These results suggested that melatonin could function as an important protective agent against the insults of whole-body irradiation. While it would be highly desirable to determine if melatonin was similarly protective against radiation damage upon human whole-body exposures, animal studies are more appropriate at this time. The current investigation, therefore, was designed to determine whether mice which were pre-treated with melatonin one hour before acute whole-body exposure to gamma radiation at the expected $LD_{50/30}$ dose exhibit a higher survival rate as compared with similarly irradiated mice which were not pre-treated with melatonin.

MATERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio. The choice of CD2-F1 mice and the dose of gamma radiation selected were based on the experience from the studies performed using other radioprotectors (23, 24).

Mice and Maintenance

Six- to eight-week-old male CD2-F1 mice were obtained from Charles River Laboratories, Wilmington, MA, USA. They were kept in an animal facility where the room temperature was maintained at 65-75°F with a relative humidity of 50-70% and an air-flow rate of 15 exchanges/hour. A time-controlled system provided 0700-2100 hour light and 2100-0700 hour dark cycles. They were given access *ad libitum* to Teklad rodent chow diet (Indianapolis, IN, USA) and water from a sanitized bottle fitted with a stopper and sipper tube. The mice were used for experimentation after a 7-day acclimatization period and were toe-clipped for individual identification.

Melatonin and Gamma Irradiation

Two separate experiments using a total of 160 mice were conducted. Each experiment had 10 mice in each of the following eight groups. A randomized block design based on body weights was used to distribute the mice into separate groups.

Group 1 Control
Group 2 815 cGy whole-body gamma radiation
Group 3 Solvent control (soybean oil)
Group 4 Solvent control + 815 cGy whole-body gamma radiation
Group 5 Melatonin (125 mg/kg body weight)
Group 6 Melatonin (125 mg/kg body weight) + 815 cGy whole-body gamma radiation
Group 7 Melatonin (250 mg/kg body weight)
Group 8 Melatonin (250 mg/kg body weight) + 815 cGy whole-body gamma radiation

Mice in groups 1, 3, 5 and 7 served as the controls for the experimental mice in groups 2, 4, 6 and 8, respectively. One hour before the start of each experiment, all mice were transferred to a laboratory near the ^{137}Cs GammaCell-40 Irradiator (Atomic Energy of Canada Ltd.) facility. Control mice in group 1 were untreated and unirradiated. Mice in group 2 were exposed to an acute whole-body gamma radiation dose of 815 cGy (the expected $LD_{50/30}$ dose). The dose rate used was 103 cGy/minute. Mice in groups 3-8 were given an intraperitoneal injection of 100 µl of either the solvent (soybean oil, Sigma, St. Louis, MO) or melatonin (Sigma, freshly prepared as a uniform suspension in soybean oil). One hour after the injections, mice in groups 4, 6 and 8 were exposed to an acute whole-body gamma radiation dose of 815 cGy. One hour after irradiation, all mice were returned to the animal facility. They were inspected twice daily, at least 6 hours apart (before 1000 and after 1400 hours) for morbidity and mortality. All mice which were alive on day 31 after radiation exposure were euthanized using halothane.

Statistical Analysis

The differences in the proportions of irradiated mice in groups 2, 4, 6, and 8 which survived through the 30-day observation period were analyzed using the Chi-Square test. The cumulative survival days of irradiated mice in groups 2, 4, 6, and 8 were also tested using the statistical methods associated with Kaplan-Meier product limit survival curves (25). The data were analyzed using Mantel-Cox Log Rank (26), Breslow (27) and Tarone-Ware (28) statistics for the differences between the survival curves, with each test placing an emphasis or weight on different areas of the curve.

RESULTS AND DISCUSSION

The numbers of mice which were alive or dead in all eight groups of two separate experiments (pooled data from both experiments) and the results of the statistical analyses are presented in Table 1. All 20 mice in each of groups 1, 3, 5 and 7 were alive at the end of the 30-day observation period. These observations indicate that the treatment of mice with solvent alone or melatonin (at as high a dose as 250 mg/kg) had no adverse effect on their survival. Only 45% of the mice in group 2, which received 815 cGy gamma radiation (the expected $LD_{50/30}$ dose), survived through the observation period. Pathological examination of the first two mice which died in this group indicated that their death could have been due to the hematopoietic syndrome. The 50% percent survival of the irradiated mice in group 4, which were pre-treated with the solvent alone, was not significantly different from the survival of mice which received 815 cGy radiation alone ($p=0.7515$). These observations indicate that the pre-treatment of mice with soybean oil did not modify their radiation-induced lethality. In contrast, pre-treatment with melatonin one hour before exposure to 815 cGy gamma radiation increased the survival of mice in groups 6 (125 mg/kg melatonin) and 8 (250 mg/kg melatonin) to 60% ($p=0.3421$) and 85% ($p=0.0080$),

respectively. These data are similar to those reported previously by Blickenstaff et al. (29), who observed an increase in the survival of male Swiss ND4 mice injected with melatonin (250 mg/kg) 30 minutes before irradiation with an acute lethal dose of 950 cGy of 6 mV photons produced by a linear accelerator. In that study, 9 of 21 mice (43%) which were pre-treated with melatonin survived over a 30-day period, while all 20 mice which were not pre-treated with melatonin died by day 12 (0% survival).

The percentage survival of the irradiated mice in groups 2, 4, 6 and 8, as a function of time over the 30-day observation period, are presented in Figure 1. Pairwise comparison showed significant differences between groups 2 and 8 (815 cGy gamma radiation alone versus 250 mg/kg melatonin + 815 cGy gamma radiation), and also between groups 4 and 8 (solvent alone + 815 cGy gamma radiation versus 250 mg/kg melatonin + 815 cGy gamma radiation) ($p=<0.05$). The difference between the mice in groups 6 and 8 (125 mg/kg melatonin + 815 cGy gamma radiation versus 250 mg/kg melatonin + 815 cGy gamma radiation) indicated a $p=0.0490$, using the Brislow statistic, while the Log Rank ($p=0.0615$) and the Tarone-Ware ($p=0.0546$) tests were less than the alpha criteria of 0.05 (Table 1).

Barchas et al. (30) conducted extensive investigations on the acute toxicological and pharmacological effects of melatonin. Toxicity tests in mice injected with melatonin doses up to 800 mg/kg failed to produce death or gross changes in the behavior; the LD_{50} dose of melatonin could not be attained because of the limitation imposed by the solubility of compound. Pharmacological studies in mice, rabbits, cats and dogs administered with melatonin (10-30 mg/kg) did not indicate any adverse effects on vascular and nervous systems. In recent studies by Vijayalaxmi et al. (31), the frequencies of sister chromatid exchanges were unaffected when mitogen-stimulated human blood lymphocytes were cultured continuously for 72 hours in the presence of 0.05-1.0 mM concentrations of melatonin, suggesting that melatonin (at the dose levels tested) was not genotoxic. Also, human volunteers who

ingested a single oral dose of 300 mg of melatonin did not report any adverse side-effects (22). In the present study, none of the 20 mice administered 250 mg/kg of melatonin died during the 30-day observation period. All of these observations provide support for the non-toxic nature of melatonin.

There is continued interest and need for the identification and development of non-toxic and effective radioprotective compounds. Such compounds could potentially protect humans against the genetic damage, mutation, immune system alterations, and teratogenic effects of toxic agents which act through the generation of free radicals. An efficient, non-toxic and orally administered radioprotector could prove useful (a) in occupational and therapeutic settings where ionizing radiation is used or exposure occurs (e.g., defense, airline, military and research personnel, nurses, dental assistants, radiotherapy and nuclear medicine technicians, etc.); (b) after nuclear accidents which leave radioactivity in the environment, such as occurred at Three Mile Island, Chernobyl and Goiania (32); and (c) during space travel to protect astronauts from the effects of high doses of radiation associated with solar flares. WR-2721 was long ago found to be an effective radioprotector. However, it has been reported to cause undesirable side-effects at the dose levels required for radioprotection and must be given intravenously. In this context, melatonin appears to be the effective and non-toxic radioprotector desired. The optimum dose of melatonin for human radioprotection, however, is yet to be determined.

ACKNOWLEDGMENTS

This study was supported by the Armed Forces Radiobiology Research Institute, Bethesda, Grant No. MDA928-97-C-0004. We thank Robert Geller for the statistical analyses of the data.

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Table 1.
Survival of mice which were injected with melatonin one hour before exposure to acute whole-body gamma radiation dose of 815 cGy (LD/50 dose).

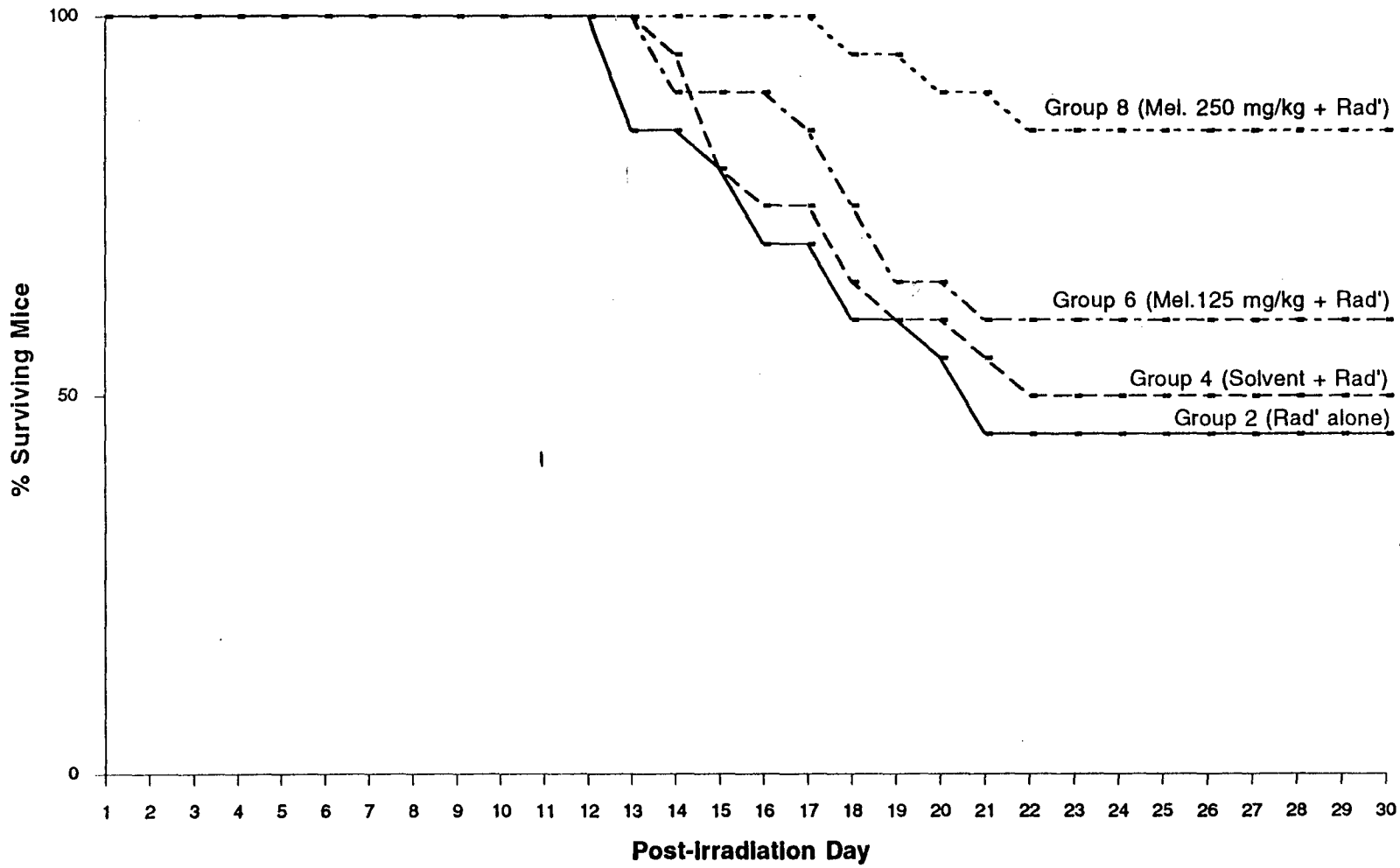
Group	Experiment 1			Experiment 2			Experiments 1 + 2			
	Total	Live	Dead	Total	Live	Dead	Total	Live *	Dead *	% Live
1. Control	10	10	0	10	10	0	20	20	0	100
2. Gamma radiation (815 cGy)	10	4	6	10	5	5	20	9	11	45
3. Solvent	10	10	0	10	10	0	20	20	0	100
4. Solvent + 815 cGy	10	5	5	10	5	5	20	10	10	50
5. Melatonin (125 mg/kg)	10	10	0	10	10	0	20	20	0	100
6. Mel (125 mg/kg) + 815 cGy	10	6	4	10	6	4	20	12	8	60
7. Melatonin (250 mg/kg)	10	10	0	10	10	0	20	20	0	100
8. Mel (250 mg/kg) + 815 cGy	10	8	2	10	9	1	20	17	3	85

Statistical Analysis:	Chi Sq.*	Log Rank**	Breslow**	Tarone-Ware**
Group 2 versus Group 4	p=0.7515	p=0.6988	p=0.6369	p=0.6665
Group 2 versus Group 6	p=0.3421	p=0.3055	p=0.2659	p=0.2849
Group 2 versus Group 8	p=0.0080	p=0.0051	p=0.0038	p=0.0044
Group 4 versus Group 6	p=0.8171	p=0.5187	p=0.5242	p=0.5207
Group 4 versus Group 8	p=0.0613	p=0.0129	p=0.0100	p=0.0113
Group 6 versus Group 8	p=0.0766	p=0.0615	p=0.0490	p=0.0546

* : Differences between the numbers of live mice.

**: Differences between the survival data presented in Figure 1.

Figure 1: Radioprotective ability of melatonin.



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4. Solvent + 815 cGy	10	5	5	10	5	5	20	10	10	50
5. Melatonin (125 mg/kg)	10	10	0	10	10	0	20	20	0	100
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